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FINAL EXAMINATION/PRIVATE DEFENSE FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN IN THE NEUROSCIENCE GRADUATE PROGRAM

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Graduate Program in Neuroscience

Uniformed Services University of the Health Sciences

Title of Dissertation: $GABA_A$ RECEPTOR-MEDIATED ACTIVITY IN A MODEL OF CORTICAL DYSPLASIA

By

Joseph Abbah, Doctor of Philosophy, 2012

Thesis directed by: Sharon L. Juliano, Ph.D.

Professor, Department of Anatomy, Physiology and Genetics, Neuroscience Program Director

ABSTRACT

Cortical dysplasia is a developmental abnormality characterized by changes in the structure and function of the neocortex. This disorder is implicated in many neuropsychiatric disorders such as epilepsy, autism and schizophrenia, and results from failure of immature neurons to appropriately migrate to their cortical targets. We developed a model of cortical dysplasia by administering methylazoxymethanol (MAM), an anti-mitotic, to pregnant ferrets on gestational day 33 leading to abnormal cortical layering, increased expression of $GABA_A$ receptors ($GABA_{AR}$) and altered distribution of neurons derived from the ganglionic eminence (GE).

We evaluated the impact of MAM treatment on the kinetic behavior of migrating GE cells and examined the relationship between changes in the ambient level of $GABA_{AR}$-mediated activity and alterations in neuronal migration

in an organotypic cultures of postnatal day 0 to 1 (P0 - P1). In addition, we investigated the functional implication of altered neuronal migration in the cortex of dysplastic animals using whole-cell patch-clamp recording and western blot assay. The normal dynamic pattern of migration of GE-derived cells alters after MAM treatment. Cells originating in the GE of normal ferrets migrate significantly faster and greater proportion of these cells demonstrates 'exploratory behavior' compared with cells in model animals. Treatment with MAM also increased the expression of $GABA_{A\alpha2}$, $GABA_{A\alpha3}$ receptor subunits and the neuron-specific potassium chloride co-transporter in the cortex of P0 – P1 animals suggesting that $GABA_{AR}$-mediated activity increases in our model animals. In support, the deficit in migration in MAM-treated GE cells improves when $GABA_{AR}$ activity is blocked.

Functional analysis showed that treatment with MAM significantly increased the amplitude and frequency of $GABA_{AR}$-mediated spontaneous inhibitory post-synaptic currents (sIPSCs) in GE cells. In older MAM-treated animals, the amplitude of sIPSCs in layer 2/3 pyramidal cells increased with no significant effect on frequency. The amplitude and frequency of miniature IPSCs, however, were significantly increased in the MAM-treated pyramidal cells. The expression of $GABA_{A\alpha3}$ receptor subunit was increased following MAM treatment. These data suggest that treatment with MAM increases GABA signaling within the neocortex, which impairs migration and distribution of GE-derived cells to alter the microcircuitry and function of the resultant cortex.

$GABA_A$ RECEPTOR-MEDIATED ACTIVITY IN A MODEL OF CORTICAL DYSPLASIA

By

Joseph Abbah

Doctoral Dissertation submitted to the Faculty of the Neuroscience Graduate Program at the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of

Doctor of Philosophy, 2012.

PREFACE

This thesis is based upon work performed in the laboratory of Dr. Sharon Juliano at the Uniformed Services University of the Health Sciences between September 3, 2007 and June 22, 2012.

DEDICATION

To my beloved wife, Faith Abbah, our beautiful son, Joseph (jnr) Owoicho Adah Joseph, and the living memory of our lovely daughter, Emily Enayi Joseph (who left us too soon to be with the Lord) - for their love and inspiration; and to the beautiful memories of my parents- Enayi Abah and Audu Abah- for making me recognize the value of education very early in life.

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This work is the product of the efforts of many individuals who made tremendous contributions in building my capacity as a professional and scientist; the tribute I pay here is an inadequate reflection of my personal sense of gratitude to them and what they have meant to me.

I am deeply indebted to Dr Sharon L. Juliano for her faith in me, for her support and mentorship, which extend beyond the confines of academia. Throughout my time in the program, she was a steady influence and guidance to me and my family. Although my journey into graduate school did not begin with my chanced meeting with her in South Africa, nonetheless, she was a major catalyst in my quest for admission; I cannot imagine under what scenario I would have ended up at USUHS had I not met her. My personal experience with her is both a reflection and a tiny representation of her dedication towards expanding the culture of neuroscience to technologically and scientifically-challenged regions of the world, sometimes at great risk to her security and life.

I want to specially thank members of my thesis committee-Dr Maria Braga (Chair), Dr Martin Doughty, Dr Joshua Corbin, Dr Xing Xiang, for their invaluable inputs, criticisms, suggestions and useful comments, which greatly improved the quality of this work.

Graduate school is a challenging task made more daunting when undertaken outside a socio-cultural comfort zone. As an international student from Nigeria, a lot of what I had to deal with in the beginning were completely

new to me and a good amount of effort was expended on adjusting rapidly to my new environment and responsibilities. This effort was aided immensely by the warmness with which I was received, the guidance and support that I enjoyed from my colleagues and faculties, and the generous outpouring of encouragement from members of the USUHS community. In particular, I like to thank my classmates- Mike Bentley, Anton Dmitriev, Nicole Flint, Nora Hibits and especially Megan Rose Hershfield for creating a very cordial class room environment into which I was seamlessly integrated. Thank you guys!

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I owe a great depth of gratitude to Dr Gamaniel Karniyus and Dr Yakubu Ngwai who recruited me to my first job and gave me the initial opportunity to deeply appreciate science. Dr Gamaniel's support and mentorship has

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Adugba, Emmanuel Oche, Elijah Abah for your love, prayers and the rich memories we shared; my sisters, Maminetu Abah, Alice Abba, Agnes Edoh, Onyema Onum, Cecilia Abah, Margaret Abah, Fatima Okodeje, Onyoibo Abah; my aunt, Aladi Abah; my in-laws, Agnes Abah, Helen Abah, Husseina Abba, Stephen Edeh, Victoria A. Edeh, Cynthia Edeh and those who, as a result of space constraints, are not mentioned here, for all you have meant to me.

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Finally, I want to thank God for making everything possible, for his faithfulness and blessings. The early phase of my life never offered any clue that I will be a high school graduate let alone attend graduate school in the US. But he made all things possible. Thank you Lord.

TABLE OF CONTENTS

LIST OF ABREVIATIONS

aCSF	Artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP	Action potential
BMI	Bicuculline methiodide
BSA	Bovine serum albumin
CGE	Caudal ganglionic eminence
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dion
CVZ	Cortical ventricular zone
DiI	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine perchlorate
E33	Embryonic day 33 (E1= day vaginal plug is seen)
GABA	Gamma (γ)-aminobutyric acid
GE	Ganglionic eminence
KCC2	Potassium- Chloride Co-transporter
LGE	Lateral ganglionic eminence
MAM	Methylazoxymethanol acetate
MGE	Medial ganglionic eminence
MK801	(5R, 10S)-(-)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclophepten-5,10-imine maleate
MZ	Marginal zone
NB media	Neurobasal media (containing B27, N2 and G1.2. supplements)
NKCCl	Sodium Potassium Chloride Co-transpoter
NMDA	N-methyl-D-aspartate

PBS	Phosphate buffered saline
RA	Retinoic acid
RG	Radial glia cells
SCH50911	(2S)-(+)-5,5-Dimethyl-2-morpholine acetic acid
SVZ	Subventricular zone
TTX	Octahydro-12-(hydroxymethyl)-2-imino-5,9:7,10a-dimethano-10a*H*-[1,3]dioxocino[6,5-*d*]pyrimidine-4,7,10,11,12-pentol citrate
VZ	Ventricular zone

CHAPTER 1

INTRODUCTION

Cortical dysplasia (CD) is a developmental abnormality characterized by changes in the structure and function of the cerebral cortex. This disorder is implicated in many neuropsychiatric disorders such as intractable epilepsy, schizophrenia, and autism, and results from failure of immature neurons to appropriately migrate and reach their cortical targets (Taylor et al., 1971; Choi and Mathias, 1987; Tassi et al., 2002; Calcagnotto and Baraban, 2003; Moroni et al., 2008). By some estimates, CD is responsible for 8-12% of all clinical cases of epilepsy that fail to respond to standard drug treatment, and 14-26% of pediatric cases of epilepsy in which surgical intervention is indicated (Li et al., 1995; Polky, 1996; Fauser et al., 2012). During development, the cerebral cortex, which consists of six distinct cortical layers, is constructed through a sequence of time-dependent processes that includes generation, migration and subsequent differentiation of two principal neuronal cell types: projection neurons, primarily pyramidal cells, and interneurons. These two major classes of neurons derive from distinct neurogenic regions of the developing cortex and use different modes of migration to reach their cortical targets (Parnavelas et al., 1991; Mione et al., 1994; Parnavelas, 2000; Nadarajah and Parnavelas, 2002; Marin and Rubenstein, 2003; Kriegstein and Noctor, 2004; Ayala et al., 2007). Over the years, studies conducted in invertebrates and rodents revealed some of the molecular mechanisms underlying the complex processes of neurogenesis and neuronal migration; these investigations demonstrate that the process of neurogenesis and neuronal migration are similar across species (Garcia-Bellido,

1979; Villares and Cabrera, 1987; Ghysen and Dambly-Chaudiere, 1988; Gonzalez et al., 1989; Jarman et al., 1993; Bertrand et al., 2002). These findings form the basis for extrapolation of developmental events observed in lower animals to higher organisms including humans. However, it is apparent from recent studies that important differences exist in the process of cortical development between lissencephallic animals such as rodents and gyrencephalic organisms, such as ferrets and primates. For instance, gyrencephalic animals such as ferrets and primates have protracted neurogenesis leading to a significant increase in population of neurons generated, as well as the size and features of the resulting cortex (Poluch et al., 2008; Fietz et al., 2010; Martinez-Cerdeno et al., 2012). In addition, the site of origin and migratory characteristics of interneurons in higher organisms differ from lower animals (Poluch et al., 2008; Letinic et al., 2002; Fietz et al., 2010). These differences underscore the need to study the process of corticogenesis under normal and diseased conditions in animals with a convoluted cortex.

Origin of Projection neurons. Projection neurons are usually characterized by a pyramidal morphology and use glutamate as their neurotransmitter. It is widely accepted that despite the unique pyramidal morphology of these cells, cortical projection neurons in different cortical layers have different molecular signatures, and are generated sequentially from stem and progenitor cells in neocortical germinal zones in the dorsal telecephalon including the ventricular zone (VZ) and the subventricular zone (SVZ) (Rakic, 1988; Walsh and Cepko, 1988, 1993). Both symmetric and asymmetric division

of stem cells in the germinal zones and their progeny generates the repertoire of projection neurons in the cortex (Noctor et al., 2001a, 2004). Neurogenesis initiates when stem cells in the neuroepithelium undergo symmetric division to generate daughter stem cells (with same molecular properties as the parent stem cells) followed by asymmetric division to produce radial glia (RG) cells (Haubensak et al., 2004; Noctor et al., 2004; Gotz and Huttner, 2005; Kriegstein and Alvarez-Buylla, 2009; Hansen et al., 2011). Within the ventricular zone (VZ), RG cells, which have stem cell properties, divide symmetrically or asymmetrically to generate daughter cells that are also RG cells, while simultaneously generating other daughter cells that represent committed neuronal progenitors. Subsequently, the progenitor cells in the subventricular zone (SZ) undergo further cycles of symmetric cell division to produce committed neuroblasts that exit the cell cycle and migrate to their final cortical target (Gray et al., 1988; Luskin et al., 1988; Price and Thurlow, 1988; Grove et al., 1993; Chen and McConnell, 1995; Kornack and Rakic, 1995; McConnell, 1995; Rakic, 1995; Mione et al., 1997; Reid et al., 1997; Qian et al., 1998 and 2000; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001a; Shen et al., 2002; Gotz et al., 2002; Fishell and Kriegstein, 2003; Kriegstein and Gotz, 2003; Noctor et al., 2004; Gotz and Huttener, 2005; Gal et al., 2006; Kriegstein and Alvarez-Buylla, 2009).

Mammals have six cortical layers populated by pyramidal cells and interneurons; the position of projection neurons is determined by the time of birth, corresponding to exit of the cell cycle (Cavines and Sidman, 1973; Cavines,

1982; Holt et al., 1988; Takahashi et al., 1999; Ohnuma and Harris, 2003). The initial group of cells migrate from the VZ; the cortical hem is also likely to provide a cohort of Cajal Retzius cells to form the preplate (Yoshida et al., 2006). This zone is split by a surge of cortical cells to create the cortical plate, now sandwiched between the more superficial MZ that includes Cajal Retzius cells and the deeper subplate layer (Marin-Padilla, 1998). Subsequent waves of cortical cells situate themselves between these two layers (in the cortical plate) in such a way that cells occupying the deep layers of 6 and 5 are born first followed by cells of the more superficial layers, described as an inside-out pattern of corticogenesis (Angevine and Sidman, 1961; Rakic, 1974; Bayer and Altman, 1991; Shen et al., 2006).

The specification and progressive generation of pyramidal cells is regulated by a molecular program involving several transcriptional factors with distinct spatial expressions. Whereas all pyramidal neurons, irrespective of layer identity, are specified by *Neurogenin 1* and *Neurogenin 2* (*Neurog1* and *Neurog2*), basic-helix-loop-helix transcriptional factors expressed in the VZ of the dorsal telencephalon (Fode et al., 2000; Bertrand et al., 2002; Ross et al., 2003; Schummanns et al., 2004; Guillemot, 2007), the molecular identity of pyramidal cells is controlled by downstream effector genes that regulate both the layer identity and axonal projections and targets of the different types of these cells (Fishell and Hanashima, 2008). For instance, projection neurons destined to the deep layers of 5 and 6 express *Sox5, Fezf2, Ctip2* and *Tbr1* and project to subcortical regions of the brain (DeFelipe and Farinas, 1992; Koster and O'leary,

1993; Kristin et al., 2002; Arlotta et al., 2005; Chen et al., 2005a, 2005b; Molyneaux et al., 2005, 2007; Chen et al., 2008). On the other hand, pyramidal cells in upper cortical layers of 2 to 4 express *Satb2* and project to intracortical regions (Britanova et al., 2008).

Migration of projection neurons. Regardless of laminar position, projection neurons born in the neocortical VZ or SVZ migrate radially, using two migratory approaches to reach their final cortical position (Marin and Rubenstein, 2003). A subset of migrating projection neurons use radial glial support and adopt 'glial guided' locomotion (Rakic, 1971, 1972; O'Rourke et al., 1992; Anton et al., 1996; Nadarajah et al., 2001). Projection neurons that use this migratory strategy attach to radial glial fibres that stretch across the entire cortical wall, as a supporting framework. Other projection neurons reach the cortical plate via a somal translocation mechanism in which cells migrate independent of radial glia support (Morest, 1970; Rakic, 1972, O'Rourke et al., 1992; Nadarajah and Parnavelas, 2002; Nadarajah et al., 2001). Migration of pyramidal cells proceeds through distinct phases that include the following steps: (1) migrating cells become physically attached to radial glia fibres (those that adopt glia-guided locomotion), (2) development and extension of a leading process, (3) nucleokinesis that involves translocation of the nucleus in the direction of the leading process (4) retraction of the trailing process and (5) detachment of cell from radial glia fiber (for glial guided migrating cells) and adoption of a cortical position (de Rouvroit and Goffinet, 2001; Honda et al., 2003; Marin and Rubenstein, 2003).

Regulation of radial migration. Each stage of the migratory process is regulated by both environmental cues and intrinsic signaling mechanisms that act in concert to modulate the cytoskeletal network of cells. Among the identified factors implicated in influencing pyramidal cell migration are motogens such as neurotrophins (NT4 and BDNF), epidermal growth factors, neurotransmitters such as GABA acting on $GABA_A$ and $GABA_B$ receptors and glutamate acting on both NMDA and AMPA receptors (Behar et al., 1996, 1998, 1999, 2000, 2001; Komuro and Rakic, 1993; Kornblum et al., 1997; Nakagawa et al., 1998; Marin and Rubenstein, 2001, 2003). Genes (e.g. *Lis1*, *Doublecortin, Filamin)*, which regulate the function of the neuronal cytoskeleton to control locomotion*,* and cues that signal migrating cells to cease locomotion and detach from radial glia cells are also crucial in regulating the migratory process (Cavinness, 1982; Reiner et al., 1993; Hattori et al., 1994; D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995; Eksioglu et al., 1996; Frotscher 1997; Sheppard and Pearlman, 1997; Dulabon et al., 2000; Feng and Walsh, 2001).

Genes including *Neurogenin 1* and *Neurogenin 2* (*Neuog1 and Neurog2*) play crucial roles in the migration of these cells (Hand et al., 2005; Ge et al., 2006; Nguyen et al., 2006). *Neurog1/2* promotes neuronal migration by inducing expression of *Dcx* and *p35,* while simultaneously repressing the activity of RhoA, a GTPase (Ge et al., 2006; Nobrega-Pereira et al., 2009). *Neurog1/2* also influence the migration of pyramidal cells by regulating (increasing) the expression of *Rnd2*, another GTPase (Nakamura et al., 2006; Heng et al., 2008).

On reaching the cortical plate, migrating cortical projection neurons assume laminar positions within the developing cortical wall and terminate migration. The ability of pyramidal cells to find an appropriate cortical target is regulated by *Brn1* and *Brn2*, POU-domain transcription factors that regulate the reelin/DAB1 pathway, and cyclin-dependent kinase (CDK5) signaling; this pathway plays critical roles in normal cortical layer formation (McEvilly et al., 2002; Sugitani et al., 2002; Nobrega-Pereira et al., 2009). Understandably, loss of function of any of the key players above due to mutation leads to abnormal cortical layering with inversion of cortex (D'Arcangelo et al., 1995; Hirotsune et al., 1995; Ogawa et al., 1995; Ohshima et al., 1996; Chae et al., 1997; Kwon and Tsai, 1998; Howell et al., 1999).

Interneurons. Cortical interneurons are a heterogeneous population of cells that originate from germinal zones under the regulatory control of a molecular program (Markram et al., 2008; Ascoli et al., 2008; Corbin and Butt, 2011). In rodents, these cells arise from three distinct germinal zones within the ventral telencephalon: the VZs of the lateral ganglionic eminence (LGE), the medial ganglionic eminence (MGE) and the caudal ganglionic eminence (CGE) (Van Eden et al., 1989; DeDiego et al., 1994; De Carlos et al., 1996; Anderson et al., 1997; Tamamaki et al., 1997; Lavdas et al., 1999; Sussel et al., 1999; Wichterle et al., 1999; Corbin et al., 2001; Letinic et al., 2002; Nery et al., 2002; Yuste, 2005; Wonders and Anderson, 2006; Batista-Brito and Fishell, 2009). An overwhelming majority of cortical interneurons are generated in the GE (Corbin et al., 2001; Wonders and Anderson, 2006). These cells express *GAD65/67* and

make up roughly 20% of the total neuronal cell population in the neocortex and use GABA as their primary neurotransmitter (Hendry et al., 1987; Meinecke and Peters, 1987; Hatten, 2002; Tamamaki et al., 2003). Similar to pyramidal cells, the concerted action of proneural genes of the bHLH motif such as *Mash1,* homeodomain transcription factors such as *Dlx1/2, Dlx 5/6, Nkx2*.1, *Nkx6.2*, *Lhx6* and *Gli1,* and other signaling molecules such as Sonic hedgehog in the GE specify and generate the different types of interneurons destined for the neocortex and non-cortical structures (Chiang et al., 1996; Anderson et al., 1997; Casarosa et al., 1999; Sussel et al., 1999; Xu et al., 2004; Butt et al., 2008; Corbin et al., 2008; Welagen and Anderson, 2011). The ability of the GE to generate diverse interneuron subtypes is due to the expression of distinct transcription factors in different regions of the GE (Butt et al., 2007; Corbin and Butt, 2011). For instance, *Gli1* and *Nkx6.1* show restricted expression in the dorsal part of the MGE (Corbin et al., 2008; Welagen and Anderson, 2011), this fact has been used to explain why the dorsal MGE is the source of somatostatin-expressing GE cells while the ventral MGE generates parvalbumin-expressing GE cells (Fogarty et al., 2007; Wonders et al., 2008), a view not supported by others (Yu et al., 2009). Similarly, *COUPTI* and *COUPTII* are expressed within the caudal ganglionic eminence and regulate the identity of cells generated within this region (Kanatani et al., 2008; Lodato et al., 2009).

Biochemically, interneurons are classified on the basis of their expression of different calcium binding proteins such as calretinin, calbindin, and parvalbumin, neuropeptide Y and somatostatin (Hendry et al., 1987; De Felipe,

1993; Fairen et al., 1984; Houser et al., 1983; Gupta et al., 2000; Hendry et al., 1989; Jones, 1975; Kawaguchi and Kubota, 1997; Lund and Lewis, 1993; Ascoli et al., 2008). Physiological classifications are based on intrinsic firing properties of the cells (Butt et al., 2005) while morphological classification relies on the distinctive appearance of the cells.

In rodents, the MGE gives rise to two broad types of interneurons: (a) somatostatin (SST) positive cells with a characteristic burst spiking firing property and a bitufted morphology, exemplified by the Martinotti cell, and (b) parvalbumin (PV) positive cells with fast spiking intrinsic properties, such as basket cells and chandelier cells (reviewed in Corbin and Butt, 2011). The cells generated in the MGE provide the bulk of cortical interneurons and also make significant contributions to interneurons in the hippocampus (Lavdas et al., 1999; Sussel et al., 1999; Witchterle et al., 1999; Pleasure et al., 2000; Anderson et al., 2001). Interneurons generated in the LGE migrate within the rostral migratory stream to reach the olfactory bulb; other LGE-derived interneurons eventually settle in the hippocampus, the striatum and amygdala (Corbin et al., 2001; Marin et al., 2000; Witchterle et al., 2001; Yun et al., 2001; Kohwi et al., 2007; Young et al., 2007; Cocas et al., 2011). The caudal ganglionic eminence (CGE) gives rise to a diverse sub-population of interneurons that express reelin, calretinin (CR), and neuropeptide Y (NPY) (Miyoshi and Fishell, 2011). These cells include the late spiking neurogliaform cells, the bipolar and tripolar cells capable of adaptation in the frequency of their firing, and the fast spiking double bouquet cells (Butt et al., 2005; Corbin and Butt, 2011).

The contribution of the different GE regions to the overall population of cortical interneurons varies across animal species. In rodents, the majority of interneurons are generated in the MGE even though a good number of these cells also originate from the lateral and caudal ganglionic eminences (Marin and Rubenstein, 2001; Lopez-Bendito et al., 2004). In ferrets, however, which demonstrates an early fusion of the LGE and MGE after midcortical development, the majority of interneurons originate from the lateral ganglionic eminence (Figure 1B; Poluch et al., 2008). In humans, many cortical interneurons are produced within the CVZ (Letinic et al., 2002; Yu and Zeevic, 2011).

Similar to pyramidal cells, the laminar position of interneurons appears dependent on the time of birth and their phenotype and reflects their source within the GE (Miller 1985; Fairen et al., 1986; Peduzzi, 1988; Leone et al., 2008). For these reasons, MGE-derived cells, which are born early during development, occupy both deep and upper cortical layers, whereas cells from the CGE, generated much later, occupy the upper cortical layers (Cavanagh and Parnavelas, 1988 and 1989; Miyoshi et al., 2007; Rymar and Sadikot, 2007; Miyoshi and Fishell, 2011). Thus the inside-out pattern of neurogenesis demonstrated by MGE-derived cells is absent in the CGE. Interestingly, interneurons and projection neurons born at a similar time period occupy the same cortical layer (Hevner et al., 2004).

Migration of cortical interneurons. The sequence of events involved in the migration of interneurons is similar to the process of migration of pyramidal cells described earlier. In general, migrating interneurons display either a bipolar

morphology with leading and trailing processes or branched leading processes (Anderson et al., 1997; Bellion et al., 2005; Friocourt et al., 2007; Kappeler et al., 2006; Lavdas et al., 1999; Marin and Rubenstein, 2001; Polleux et al., 2002) Martini et al., 2009). Migration is achieved with a step-wise development and extension of a leading process followed by nucleokinesis and retraction of a trailing process (Marin and Rubenstein, 2001, 2003). Interneurons travelling to the neocortex migrate through multiple routes and complex pathways. Interneurons first migrate within the subpallium towards the corticostriatal border; the path taken by GE-derived cells within this region is influenced by the time of their birth. In general, interneurons fated to lower cortical layers (early born) migrate within the IZ of the GE where as upper cortical layers (late-born) interneurons migrate through the VZ/SVZ of the striatum before entering the neocortex (Anderson et al., 2001; Ang et al., 2003). As they cross the corticostriatal boundary, migrating GE-derived cells, which are oriented tangentially, use two major routes to reach the neocortex: a subset of interneurons, particularly those originating from the MGE, migrate through the IZ and SVZ; other MGE-derived cells travel through the MZ and the preplate (Anton et al., 1996; Kriegstein and Noctor, 2004; Poluch and Juliano, 2008; Lavdas et al., 1999; Anderson et al., 2001; Wichterle et al., 2001; Jimenez et al., 2002; Ang et al., 2003; Tanaka et al., 2003). LGE-derived cells are also believed to travel tangentially within the SVZ (Anderson et al., 2001).

On reaching the neocortex, migrating interneurons (in the IZ/SVZ and MZ) switch their orientation from tangential to radial mode to reach the cortical plate

and take up a laminar position (Anderson et al., 2001; Nadarajah et al., 2001; Anderson et al., 2002; Nadarajah and Parnavelas, 2002; Wichterle et al., 2001; 2003; Ang et al., 2003; Tanaka et al., 2003, 2006; Poluch and Juliano 2007, 2008). Some interneurons that migrate tangentially within the IZ/SVZ switch to radial orientation and migrate past the cortical plate to reach the MZ before reversing direction to migrate downwards to enter the cortical plate (Tanaka et al., 2003). It has also been observed that populations of interneurons exhibit a 'ventricle-directed' migration. These cells, after reaching the neocortex, reverse direction and migrate to the ventricle before proceeding to migrate back to the cortical plate (Nadarajah et al., 2002). Various reports suggest that the radial phase of interneuron migration use radial glia or axonal support (O'Rourke et al., 1995; Denaxa et al., 2001; Letinic et al., 2002; Polleux et al., 2002; Nadarajah et al., 2002; Ang et al., 2003; Poluch and Juliano, 2007; Yokota et al., 2007).

Functionally, interneurons play important roles in local cortical circuits and in modulating the excitatory output of pyramidal neurons (Cobb et al., 1995; Thomson et al., 1996; Anderson et al., 1997; Wichterle et al., 1999; Parnavelas, 2000; Tamas et al., 2000; Marin and Rubenstein, 2001).

Regulation of interneuron migration. As mentioned earlier, in addition to cortical interneurons, the GE also provides interneurons to other telencephalic structures (such as the hippocampus, striatum and olfactory bulb), which raises the question as to what mechanisms ensure appropriate migration of these cells to their ultimate target destinations. Although the full picture has yet to emerge, available evidence suggests that the combination of intrinsic mechanisms and

environmental cues ensure guided migration of GE-derived cells to their final targets (Figure 1A, Mason et al., 2001; Marin et al., 2003; Wichterle et al., 2003; Corbin and Butt, 2011). The factors responsible for guiding interneurons from the GE to their various targets can be roughly classified into: (a) motogenic factors (b) repulsive signals (c) attractants (d) neurotransmistters (e) genetic factors and (f) stop signals (Marin and Rubenstein, 2001; 2003; Ayala et al., 2007; Corbin and Butt, 2011). Motogenic factors trigger kinetic activity in GE-derived cells leading to random movement in all directions. For instance, hepatocyte growth factor (HGF), a motogen, acts on μ-PAR receptors to selectively promote the migration of parvalbumin-positive interneurons to the neocortex (Powell et al., 2001). Thus, in loss-of-function experiments such as in μ-PAR-/- mutants, interneuron migration is impaired leading to significant reduction in GE-derived cells in the neocortex. Neurotrophins such as NT4 and BDNF activate TrkB neurotrophin receptors to stimulate migration of GE cells, which underscores the migration deficit seen when neurotrophin signaling is impaired (Brunstrom et al., 1997; Polleux et al., 2002).

Repulsive signals regulate the movement of GE-derived cells to cross the cortico-striatal boundary enroute to the neocortex. The ability of cortical interneurons to sort out from GE-derived cells destined to the striatum is in part due to the repellant action of semaphorins mediated by semaphorin-neuropillin signaling. Cortical interneurons express two transmembrane receptors, neuropillin 1 and neuropilin 2 (*Nrp1/2*), enabling them to respond to repulsive signals of semaphorin 3A and semaphorin 3AF expressed in the striatum to thus

avoid settling in the striatum (Marin et al., 2001b; Tamamaki et al., 2003). Other studies suggest a role for Slit proteins (*Slit1* and *Slit2*), which are expressed in the subpallium during development, and *Netrin* as repulsive signals to migrating interneurons, but this claim is undermined by observations in *Slit1* and *Slit2* double mutants or in *Slit1* and *Slit2* and *Netrin1* deficient animals, in which the number of cortical interneurons were not significantly altered (Zhu et al., 1999; Hamasaki et al., 2001; Marin et al., 2003; Wichterle et al., 2003). Recently, it has been suggested that repulsive signals expressed in the cortical hem regulate the switch in orientation of migrating GE cells from tangential to radial as cells migrate to the cortical plate (Caronina-Brown and Grove, 2011).

In various *in-vitro* migration assays, migrating interneurons are attracted to cortical cells as well as ectopically placed cortical explants, suggesting that neocortical diffusible attractive factors modulate the directional movement of interneurons (Marin et al., 2003; Poluch et al., 2008). This suggestion is supported by observations that genetic disruption of embryonic cortex in *Emx1* and *Emx2* double mutants impairs the migration of interneurons, indicating that factors intrinsic to the cortex influence interneuron migration (Shinozaki et al., 2002). Furthermore, when normal and dysplastic GE explants are placed together in coculture experiments, improved orientation of interneurons migrating toward the normal cortex indicate that the neocortical environment plays an important role in the proper migration of interneurons (Poluch et al., 2008). Taken together, these observations provide strong evidence that the neocortical environment is a critical determinant of proper migration of GE-derived neuronal

cells destined for the cortex. However, the identities of candidate neocortical cues that modulate interneuron migration are yet to be conclusively established. Nonetheless, there have been suggestions that GDNF and Neuregullin (Flames et al., 2004; Pozas and Ibanez, 2005; Ghashghaei et al., 2006), and chemokines via CXCR7 and CXCR4 receptors (Stumm et al., 2003; Lopez-Bendito et al., 2008; Sanchiz-Alcaniz et al., 2011; Wang et al., 2011) are chemoatttractant to migrating GE cells.

Neurotransmitters also regulate migration of GE-derived neurons. Migrating interneurons express AMPA, NMDA, $GABA_A$ and $GABA_B$ and dopamine receptors and may therefore respond to activity mediated by these molecules (Lopez-Bendito et al., 2003; Represa and Ben-Ari, 2005; Manent et al., 2006; Crandall et al., 2007). Indeed, the role of these agents as modulators of interneuron migration has been demonstrated in a number of migratory models in which the actions of these agents operated via a Ca^{2+}-dependent mechanism (Behar et al., 1996, 1998, 1999, 2001; Komuro and Rakic, 1992, 1993; Metin et al., 2000; Poluch and Konig, 2002; Poluch et al., 2008; Soria and Valdemolmillos, 2002).

Proneural genes also play important roles in the specification of interneurons and regulate migration of these cells. For instance, the homeodomain transcription factor, *Nkx2.1,* expressed in the MGE, negatively regulates the expression of *Nrp* receptors in migrating GE-derived cells that control the sorting of cortical interneurons (Nobrega-Pereira et al., 2008). Thus, in the presence of *Nkx2.1*, *Nrp* is downregulated, resulting in the failure of MGE

cells to respond to semaphorin signaling leading to the reduction of interneurons entering the neocortex (Nobrega-Pereira et al., 2008, Nobrega-Pereira and Marin, 2009). A similar role has been suggested for the homeobox transcription factor, *Dlx1/2* in regulating interneuron migration, presumably by controlling the expression of *Nrp* receptors and *Arx* (Le et al., 2007; Cobos et al., 2005a, 2005b, 2007). The aristaless related homeobox gene, *Arx*, has been implicated in modulating migration of interneurons both in rodents and humans (Kitamura et al., 2002; Alifragis et al., 2004; Liodis et al., 2007; Friocourt et al., 2008).

Retinoic acid (RA) also influence the migration of cells leaving the GE (Crandall et al., 2011). RA inhibits GE cell migration probably through regulation of dopamine D2 receptor expression. Activation of dopamine D2 receptors inhibits GE cell migration (Crandall et al., 2007). Consequently, blocking D2-receptors abolishes the effect of RA leading to enhancement of GE cell migration. Recently, it was demonstrated that a subset of interneurons that are generated in the CGE depend on activity for migration and development of axonal arbors for network integration (Natalia et al., 2011).

On reaching their target destination in the cortex, migrating GE cells terminate migration and take up a cortical position. How this is achieved still remains unclear. Local signals from projection neurons within the same cortical layer and/or from the meninges may instruct migrating GE cells to take up cortical position (Tiveron et al., 2006; Li et al., 2008; Li and Pleasure, 2011; Lodato et al., 2011; Caronia-Brown and Grove, 2011; Zarbalis et al., 2012). The neuron-specific potassium- chloride co-transporter, KCC2, has also been found

to act as a stop signal (Bortone and Polleux, 2010) (for detailed discussion on this topic, see below).

Role of GABA in cortical development and function. Within the cerebral cortex of adult mammals, inhibitory neurotransmission is mediated primarily by GABA, through two receptor subtypes: the fast conducting ionotropic $GABA_A$ receptors and the slow conducting metabotropic $GABA_B$ receptor (Tija et al., 2008). Thus GABA signaling modulates excitatory outputs of glutamatergic projection neurons to establish a balance between excitation and inhibition to ensure normal cortical function (Gilbert and Wiesel 1985; Jones 1986; Cobb et al., 1995; Thomson et al., 1996; Tamas et al., 2000; Freund, 2003; Hensch, 2005; Somogyi and Klausberger 2005; Caronia-Brown and Grove, 2011). Consequently, alterations in GABAergic signaling have been implicated in many neuropsychiatric disorders such as epilepsy (Luhmann et al., 1995; Loup et al., 2000; Kobayashi and Buckmaster, 2003; Fristch et al., 2009; Gant et al., 2009; Kang and Barnes; 2012; Ma and Prince et al., 2012), Alzheimers Disease (Young, 1987; Fonseca et al., 1993; Lanctot et al., 2004; Limon et al., 2012), schizophrenia (Keverne, 1999; Hashimoto et al., 2003; Lewis et al., 2004, 2005; Bernstein et al., 2007; Chattopaddhyaya and Di Cristo, 2012; Moyer et al., 2012**)** autism (Fatemi et al., 2006 , 2009; Chao et al., 2010; Oblak et al., 2010; Chattopaddhyaya and Di Cristo, 2012; Kang and Barnes; 2012), and X-linked lissencephaly with abnormal genitalia (Colombo et al., 2007).

Interestingly, the role of GABA as an inhibitory neurotransmitter is not innate but evolves. During development of the neocortex, migrating neurons first

express GABA receptors and receive signals from striatally released GABA, followed by subsequent activation of excitatory glutamate receptors (Owens et al., 1999; Tyzio et al., 1999; Hennou et al., 2002). There is good evidence that GABA plays a multifaceted role during development, in regulating DNA synthesis, cell proliferation, migration, differentiation, development of AMPA receptors, excitatory synapse formation and possibly acting as a stop signal for migrating GE cells (Barbin et al., 1993; Heck et al., 2007; LoTurco et al., 1995; Wang and Kriegstein, 2008, 2009; Bortone and Polleux, 2010; Inada et al., 2011). It has been known for several years that GABA acts as an excitatory neurotransmitter early in development to facilitate cell migration, but switches to mediate inhibition as cells mature (Plotkin et al., 1997; Clayton et al., 1998; Rivera et al., 1999). The dual role of GABA is attributed to the temporal expression of two cation-chloride transporters, the sodium-potassium-chloride co-transporter (NKCCl) and the potassium-chloride co-transporter (KCC2). Migrating neurons initially express NKCCl, which imports chloride ions (Cl^-) into the cell to maintain a higher intracellular Cl^- concentration (Clayton et al., 1998). In response to $GABA_A$ activation, the outward movement of Cl^- results in depolarization of the cells and triggers Ca^{2+}-mediated events that influence the cytoskeleton and stimulate migration (Behar et al., 2001; Soria & Valdeolmillos, 2002). As cells mature, NKCC1 downregulates and coincides with increased expression of KCC2 (Plotkin et al., 1997; Delpire, 2000; Li et al., 2002; Owens and Kriegstein, 2002). KCC2 is a Cl^- exporter that maintains a higher extracellular gradient of Cl^-. Consequently, $GABA_{AR}$ activation when KCC2 is

increased results in hyperpolarization and cell inhibition (Payne, 1997; Rivera et al., 1999). In addition, GABA activity is believed to be an important factor in promoting the expression of KCC2 during development (Ganguly et al., 1999).

The role of GABA in neuronal migration has been evaluated in various *in vitro* and *in vivo* migratory models, with researchers finding both enhancement and inhibition of neuronal migration (Behar et al., 1996, 1998, 1999, 2001, Heck et al., 2007). These discrepancies may arise from differences in the composition of cell type and the design of the various studies. In addition, many experiments did not distinguish between effects elicited on cells arising from the GE vs CVZ. The dose of GABAergic agonists and antagonists appears to have differential effects, as low concentrations of GABA cause directed migration, whereas high concentrations appear to cause random movement (Behar et al., 1996; 1998). In a study by Heck et al. (2007) both $GABA_{AR}$ agonists and antagonists delivered by elvax result in ectopic migration in the neocortex in neonatal animals. Bolteus and Bordey (2004) demonstrated that enhancement of GABAergic activity in acute brain slices slows the rate of cellular migration; although this study evaluated chain migration in the olfactory system, which may use other mechanisms than interneurons migrating into the neocortex. In addition to differences in cell types studied and the type of assay used, the seemingly complex and contradictory role of GABA in modulating migration of interneurons could also be due in part to the dynamic and temporal expression of the different subsets of GABA receptors in migrating GE cells, which determines the nature of response of these cells to GABA (Laurie et al., 1992; Poulter et al., 1992;

Fritschy et al., 1994; Cuzon et al., 2006; Wang and Kriegstein, 2009; Carlson and Yeh, 2010). Taken together, these studies indicate that GABA has an effect on neuronal migration, but the exact process needs continued clarification.

In addition to affecting migration, GABA, as a result of KCC2 expression, can also act as a stop signal for migrating GE cells (Bortone and Polleux, 2010). The effect of GABA on neuronal migration is also likely to be dependent on Ca^{2+}. In the neocortex, stimulation of $GABA_A$ receptors activates changes in intracellular calcium levels in tangentially migrating cells and this effect may influence growth cone dynamics and thus migratory behavior (Behar et al., 2001; Soria & Valdeolmillos, 2002).

The excitatory action of GABA during early neocortical development (Ben-Ari et al., 1989; Cherubini et al., 1991) is also implicated in the establishment of AMPA receptors and formation of synapses- an effect mediated via NMDA signaling (Wang and Kriegstein, 2008). Recently, our lab demonstrated that $GABA_{AR}$ mechanisms are important for both the migration and orientation of tangentially migrating interneurons (Poluch et al., 2008). Taken together, these observations underline an important role of GABA signaling during migration and cortical development.

Cortical dysplasia. Perturbation in the process of cell generation, migration or terminal differentiation as a result of genetic or environmental factors can profoundly disrupt corticogenesis leading to cortical dysplasia. These aberrations, which are often associated with abnormal GABA signaling (Rosen et al., 1998; Roper et al., 1999; Talos et al., 2012; Cepeda et al., 2012), underlie a

vast number of neurological/neuropsychiatric disorders including drug-resistant epilepsies requiring surgery, depression and schizophrenia (Chugani et al., 1990; Palmini et al., 1991; Gleeson and Walsh, 2000; Zhu and Roper, 2000; Ross and Walsh, 2001; Calcagnotto et al., 2002; Calcagnotto and Baraban, 2003; Colombo et al., 2003; Lerner et al., 2009). The etiological factors underlying the development of cortical dysplasia may either be genetic or environmental in nature. Among the identified genetic factors are mutations in key genes that play a pivotal role in neuronal migration such as *Lis1* (which results in lissencephaly), and *Doublecortin* (that leads to double cortex in females and heterotopias in males) (des Portes et al., 1998; Feng and Walsh, 2001; Gleeson et al., 1998; 1999). Prenatal alcohol exposure, carbon monoxide intoxication from cigarette smoke, alcohol intoxication are known environmental factors mediating cortical dysplasia (Ginsburg and Myers, 1974a and b; Choi and Matthias, 1987; Barinaga, 1993; Komatsu et al., 2001; Gorman et al., 2001; Autti-Ramo et al., 2002).

MAM Model of Cortical dysplasia: In order to gain critical insight into the underlying pathology in cortical dysplasia, many animal models of the syndrome have been developed to mirror specific deficits of the disorder observed in humans. Genetic models are generated through manipulation of key genes involved in cortical development such as *Lis1*, *Doublecortin, Reelin, Otx1, TSC1 cKO;* this generates phenotypes such as smooth brain, double cortex, reeler mouse, flathead, Tish, and Eker rats (Caviness & Yorke, 1976; Acampora et al., 1996; Lee et al., 1997; Yeung et al., 1997; Hirotsune et al., 1995, 1998; Roberts

et al., 2000; Uhlmann et al., 2002; Moroni et al., 2008). Other models leading to CD not involving genetic manipulation, such as freeze lesions and cortical undercut, rely on physical assault to the developing brain to induce injury and subsequent aberrant migration (Rosen et al., 1992 and 1998; Jacobs et al., 1996). In addition, some models use toxins such as carmustine, MAM, and radiation to disrupt cortical development in-utero in rodents (Ferrer, 1993; Ferrer et al., 1993; Roper, 1998; Colacitti et al., 1999; Benardete and Kriegstein, 2002; Moroni et al., 2008).

MAM is an antimitotic that transiently interferes with the cell cycle to inhibit neurogenesis. MAM methylates the 7' position of the guanine residue, which results in inhibition of DNA polymerase activity, and thus reduces nucleic acid synthesis (Matsumato and Higa, 1966). Our laboratory developed a model of cortical dysplasia, in which we administer MAM intraperitoneally to pregnant ferrets at specific gestational periods. Ferrets are the smallest mammal with a gyrencephalic cortex and thus make excellent models for studying development of the neocortex. The presence of sulci and gyri is evolutionarily significant since these features, which expand the cortical surface area, enhance the capacity for higher cognitive function (Kriegstein et al., 2006). These features are present in humans and underlie the need to develop and study this disorder in more advanced animal models.

MAM transiently disrupts cell production resulting in different types of cortical dysplasia depending on the time of administration (Noctor et al., 1999; Gierdalski and Juliano., 2003; Hasling et al., 2003; Gierdalski and Juliano, 2005;

Poluch and Juliano, 2007, 2010). Administration of MAM during the birth of layer 4 (E33) results in diminished or near total loss of this layer and widespread terminations of thalamic afferents in the neocortex (Figure 3A; Noctor et al., 1999, 2001b; Palmer et al., 2001). Of significance, ferrets treated with MAM on E33 demonstrate aberrant distribution of GABAergic interneurons (Poluch et al., 2008), increased expression of $GABA_{AR}$ in the cortex (Figure 3B; Jablonska et al, 2005), alteration in the balance of excitation and inhibition, and overall changes in the pattern of cortical information transfer (Jablonska et al., 2004; McLaughlin and Juliano, 2005; Noctor et al., 2001b). Although the morphology and overall number of GABAergic interneurons in the neocortex is not altered, the laminar position of certain categories of interneurons is changed in the E33 MAM model, suggesting that these neurons do not migrate into their proper target sites (Poluch et al., 2008). An important question that arises from this finding of aberrant interneuron redistribution is whether they occur as a result of changes in the kinetic behavior of migrating cells due to intrinsic mechanisms, or as a result of changes in the neocortical environment, or both. Ferrets treated with MAM display an altered distribution of subsets of interneurons and functional deficits similar to those seen in other models of cortical dysplasia (Rosen et al., 1998; Palmer et al., 2001; Cacagnotto et al., 2002; McLaughlin et al., 2005; Poluch et al., 2008). Although we do not observe clinical symptoms of epilepsy in our model as has been reported following similar treatments in rats (Baraban and Schwartzkroin, 1996; Chevassus-au-Louis et al., 1998; de Feo et al., 1995; Germano et al., 1996; Kimberle et al., 1999), the aberrant redistribution and

position of interneurons seen in our model raises the likelihood that the inhibitory function of these cells might be compromised, which may impair overall cortical function. This study investigates the impact of MAM treatment on the dynamic pattern of migration of GE cells as it relates to the basal level of $GABA_A$ activity and function of the resulting cortex.

A **B**

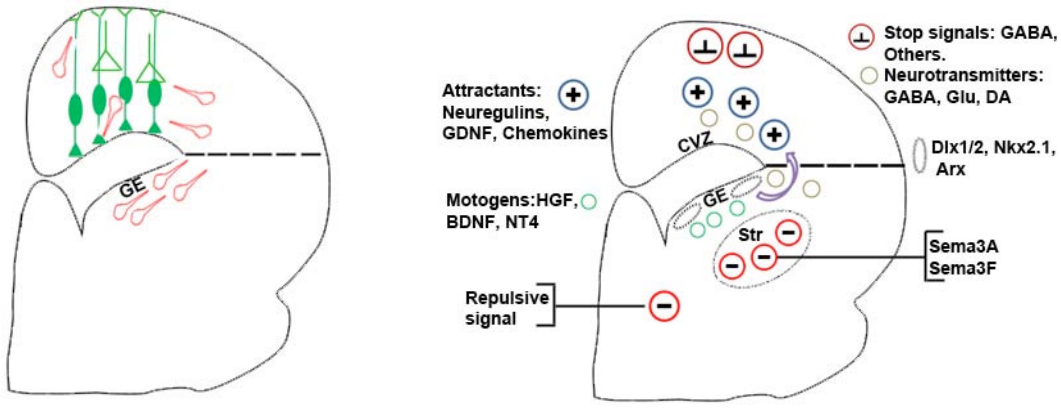

Figure 1: (A). During development, projection neurons and interneurons are generated from distinct neurogenic regions. Projection neurons form in the cortical ventricular zone and migrate radially to the cortical plate. Cortical interneurons are generated in the ganglionic eminences (GE) but migrate tangentially and radially to settle in the cortex. (B) Several extrinsic cues, including GABA, act in concert with intrinsic signaling mechanisms to regulate the process of migration of interneurons. The anatomic layout of the GE varies across species. Unlike rodents with three distinct subtypes of GE, the GE in ferrets fuses at midcortical development. Broken lines signify the corticostriatal boundary.

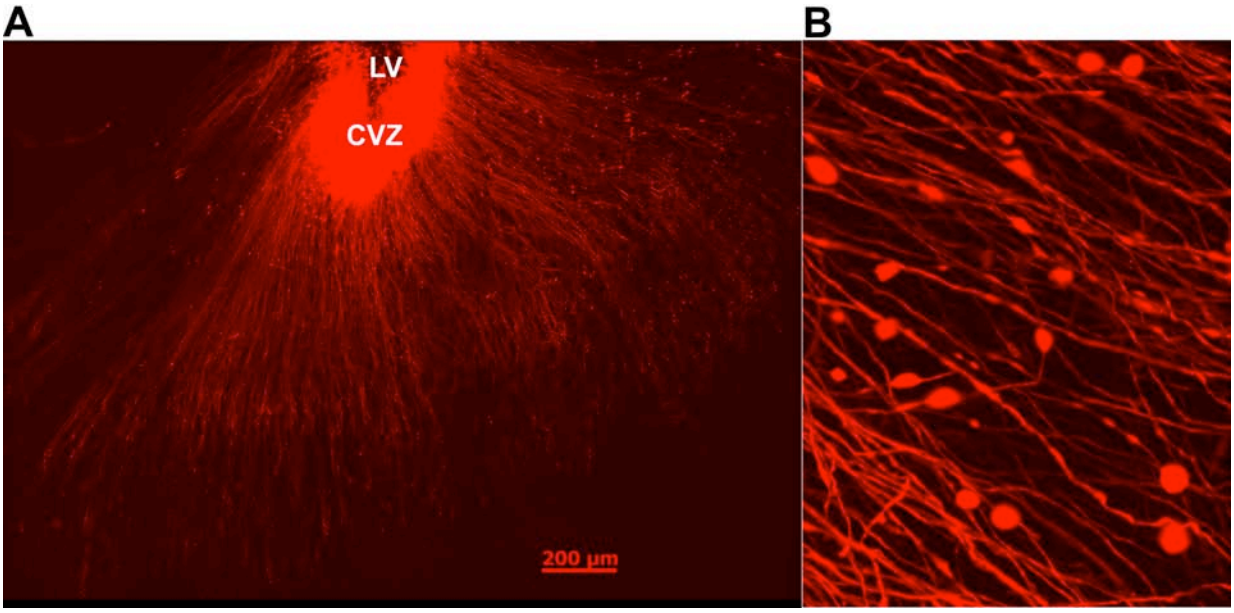

Figure 2: Pyramidal cells are generated from cortical ventricular zone and migrate radially to different cortical layers. In this picture, cells within the CVZ were electroporated with a plasmid producing red fluorescent protein. (A) Labeled radial glia cells that span the cortex can be seen aposed with migrating projection neurons (B) High power view of a section of migrating projection neurons on radial glial cells.

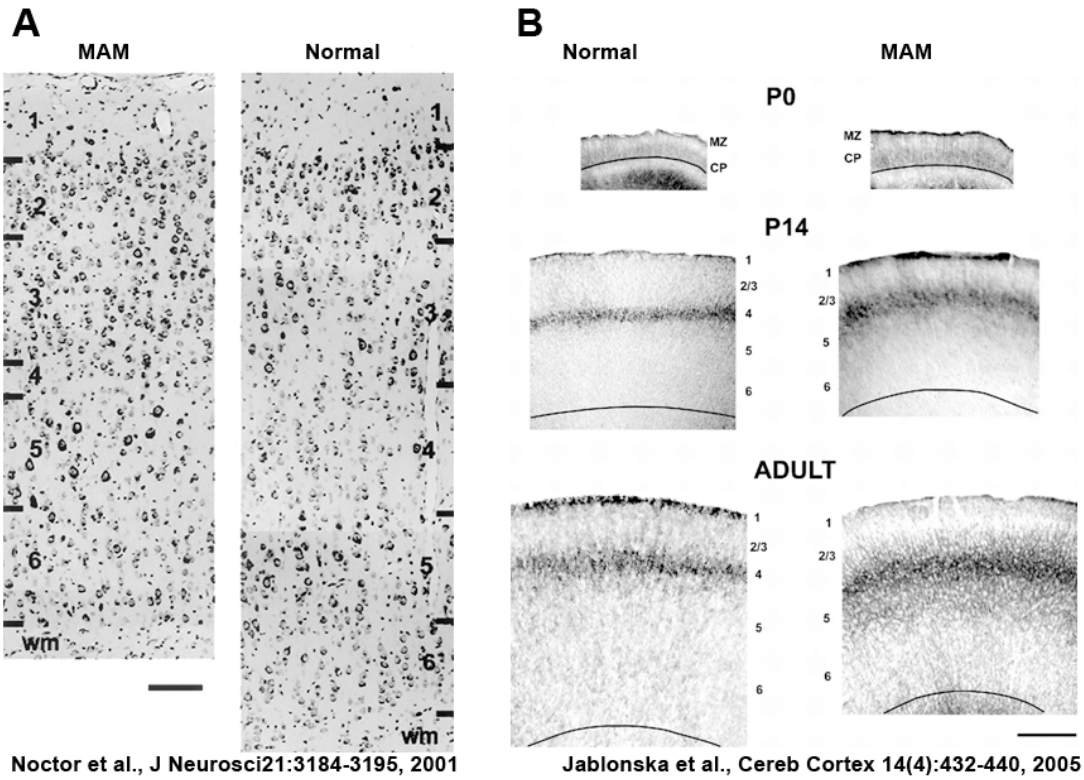

Figure 3: Administration of MAM on E33 induces several developmental malformations on the cortex. (A) As a result of transient inhibition of neurogenesis, layer 4 cells, which are born during this time period, are not generated leading to reduction in the thickness of cortical layer 4. (B) After the reduction in thickness of layer 4, $GABA_A$ receptors, which are normally expressed in high density in this region, expand to upper cortical layers.

CHAPTER 2

Altered kinetic behavior underlies redistribution of interneurons in a model of cortical dysplasia: the influence of elevated $GABA_A$ receptor activity.

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Running Title: GABA activity is critical for migration of GE cells

Abstract

Appropriate function of the neocortex depends on timely generation and migration of cells produced in the germinal zones of the neocortex and ganglionic eminence (GE). Failure to accurately complete migration results in cortical dysplasia, a developmental syndrome implicated in many neurologic disorders. We developed a model of cortical dysplasia in ferrets involving administration of methylaxozymethanol acetate (MAM), an antimitotic, to pregnant ferrets on gestational day 33, leading to dramatic reduction of layer 4 in the neocortex. Here, using time-lapse video imaging, we investigate kinetic behavior of migrating GE cells in ferrets and the role of $GABA_A$ activity. Treatment with MAM significantly reduced migration speed and the relative proportion of GE cells demonstrating exploratory behavior. Pharmacologic inhibition of $GABA_A$ receptors ($GABA_{AR}$) improved the speed of migration and exploratory ability of migrating MAM-treated GE cells. Additionally, the expression of α2 and α3 subunits of $GABA_{AR}$ and the potassium chloride co-transporter (KCC2) increased in the neocortex of MAM-treated animals. After MAM treatment, increases in endogenous KCC2 and $GABA_{AR}$ combine to alter the kinetic properties and exploratory behavior of migrating interneurons in ferrets. We show a direct correlation between increased $GABA_A$ and KCC2 expression with impaired migration and ability to explore the environment.

Keywords: development, ferret, KCC2, MAM, neuronal migration

Introduction

The laminar organization of the neocortex and its ability to function depends on timely generation and proper migration of cells originating from the ventricular zones of the neocortex (CVZ) and GE. Events that adversely impact the migratory process can lead to cortical dysplasia, a developmental abnormality characterized by aberrant cell clustering, altered gyral patterns, and changes in electrophysiological profile (Taylor et al. 1971; Choi and Mathias 1987; Tassi et al. 2002; Calcagnotto and Baraban 2003; Moroni et al. 2008). The aberrations underlie a vast number of neurological/neuropsychiatric disorders including drug-resistant epilepsy, depression, and schizophrenia (Palmini et al. 1991; Gleeson and Walsh 2000; Zhu and Roper 2000; Ross and Walsh 2001; Calcagnotto et al. 2002). Understanding the process of neuronal migration into the neocortex is critical to comprehending the many disorders that result from disrupted migration.

We developed a model of cortical dysplasia by administering a short acting anti-mitotic, methylazoxy methanol (MAM), to pregnant ferrets on gestational day (E33). Ferrets are the smallest animal with a convoluted cortex, which make them an important model for neocortical development. The tangential expansion of the neocortex, which assists in the formation of the sulci and gyri, relies on proliferation of outer subventricular progenitors, a cell population found in ferrets and humans, but absent in rodents (Fietz et al. 2010; Lui et al. 2011; also see Martinez et al 2012 for a different point of view). Our lab also found distinctions in the migratory patterns of interneurons in rodents and

ferrets, indicating the importance of studying a more developed model of neocortex (Poluch et al. 2008).

Treatment with MAM on E33 coincides with the generation of layer 4 and results in its dramatic reduction and in widespread re-distribution of $GABA_{A\alpha R}$ (Noctor et al. 1999; Palmer et al. 2001; Jablonska et al. 2004). Although the morphology and overall number of interneurons in the neocortex is not altered, the laminar position and orientation of interneurons is changed in the MAM model, suggesting that these neurons do not migrate into their proper target sites (Poluch et al. 2008). The aberrant redistribution of interneurons and $GABA_{A\alpha R}$ within the neocortex of MAM-treated animals raises important questions. 1) Do changes in the distribution of cells arising from the GE occur as a result of altered kinetic behavior of migrating GE cells, and, if so 2) are these changes influenced by the ambient activity of GABA?

Using real-time video imaging and an *in-vitro* migration assay of organotypic cultures of neonatal ferrets, we observed the dynamic movement patterns of neurons leaving the GE and CVZ. Treatment with MAM impairs the speed and exploratory potential of tangentially migrating interneurons leaving the GE without any significant effects on neurons arising from the CVZ. GE cells in MAM-treated animals exposed to GABA antagonists showed a significant improvement in the speed of migration and the proportion of cells that display exploratory activity. This reinforces the idea that E33 MAM treatment alters the migration kinetics of GE cells. The changes in the dynamic movement of GE cells are related to abnormal levels of $GABA_A$-mediated activity, which diminishes the

capacity of these cells to explore the environment and effectively migrate to their target within the neocortex.

Materials and Methods

Timed pregnant ferrets were obtained from Marshal Farms (New Rose, NY) and maintained in the animal facilities of the Uniformed Services University of the Health Sciences (USUHS). Pregnant ferrets were injected with 14mg/kg of MAM (Midwest Research Institute, Kansas City, MO) IP on E33 under isofluorane anesthesia (1-2%). After recovery from anesthesia, ferrets were maintained in the animal facility until their kits were delivered. Handling of animals complied with the USUHS Institutional Animal Care and Use Committee policy on the humane use and treatment of animals.

Preparation of Organotypic slices: Preparation of organotypic cultures was accomplished as previously described (Palmer et al. 2001). Postnatal day 0 to day 1 (P0-P1) ferret kits were anesthetized with sodium pentobarbital (50mg/kg), their brains removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM): 124 NaCl, 3.2 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4 and 10 glucose bubbled with 95% O_2 and 5% CO_2 under a laminar flow hood. Coronal slices (350-500 μm thick) were prepared from each hemisphere using a tissue chopper (Stoelting Co., Wood Dale, Illinois). Brain slices were transferred into 0.4 μm culture plate inserts (Millicell-CM, Bedford, MA) placed in six-well plates containing Neurobasal media with B27, N2, and G1.2 (containing gentamycin and glutamine) supplements. Slices were incubated at 37°C under 95% O_2 and 5% CO_2. In some experiments, bicuculline methiodide (BMI; Tocris Bioscience, Park Ellisville, MO) at a final concentration of 10 μM was added to the media.

Cell labeling: Two approaches were used to label migrating neurons. In one approach, crystals of 1,l'-dioctadecyl-3,3,3_,3_-tetramethylindocarbocyanine percholate (DiI, Invitrogen, Carlsbad, CA) were placed in the GE or CVZ using pulled borosilicate glass pipettes. In another approach, we used electroporation to focally transfect cells within the ventricular zone (VZ) of the GE using a modification of the method described by Flames et al, 2004. Transfection was accomplished using a plasmid that codes for red fluorescent protein (RFP), which was cloned into pCAGGS expression vector (a gift from Dr Tarik Haydar). Between 1-3 µl of plasmid DNA (2.7-3.6 µg/µl) was injected into the VZ of the GE of organotypic slices of ferrets. The cathode of a gene paddle electrode (Harvard Apparatus, Inc., Holliston, MA) was placed within the lateral ventricle close to the VZ of the GE while the anode was placed closed to the pial surface in an appropriate position. A pulse of 60V was applied four times, each lasting for 50 ms at intervals of 950 ms using a BTX ECM830 pulse generator (Gal et al 2006; Harvard Apparatus, Inc., Holliston, MA). Slices were incubated as above for at least 24 hrs prior to video imaging.

Video imaging: After incubating organotypic slices for 24 h (CVZ-derived) or 48 h (GE-derived) for analysis, migrating neurons were continuously visualized using an Axiovert 200 inverted microscope fitted with an apotome and Axiovision software (Carl Zeiss AG, Oberkochen, Germany). The microscope was fitted with an incubation chamber and a holder for the slices, which were maintained with humidification at 37°C with 95% O_2 and 5% CO_2. Serial stacks of images taken through the thickness of the slice were collected using a 10X objective every 30

min for 24 hrs. The image stacks were collapsed into a single frame prior to analysis of migration. Migrating GE cells were analyzed after crossing the corticostriatal boundary into the neocortex.

Analysis of migration: To measure speed, migrating cells captured in real time were tracked using ImageJ software (http://rsb.info.nih.go/ij) and the tracked distances measured and expressed over time (to obtain the speed in real time) using a program written in R statistical language (R Development, 2009). Also, the orientation of migrating GE cells (either tangential or radial) and exploratory activities were quantified for each migrating cell. The distribution of migrating GE cells within the neocortex in both normal and MAM-treated animals was determined using Axiovision software. To do this, the neocortex was divided into three roughly concentric regions (0-350 μm, 350-700 μm and >700 μm) from the border of the GE VZ and labeled neurons found within the various bins were counted and expressed as a percentage of the total number of cells.

Western Blot analysis: The neocortex was dissected from organotypic slices of normal and MAM-treated P0-P1 ferret brains (prepared as described above), frozen on dry ice and preserved at -82°C prior to use. Tissues were homogenized using RIPA lysis buffer (Santa Cruz Biotech, Santa Cruz, CA) followed by centrifugation at 14,000 g at 4°C. Protein concentration was estimated using a colorimetric assay. Proteins were separated by SDS-PAGE using 10% Bis-Tris gel and electrophoretically transferred to a PVDF membrane (Invitrogen, Carlsbad, CA). A loading volume of 10 μl containing 1-2 μg of protein was used for each analysis. Membranes were incubated with Casein blocking

buffer (PBS (0.5M NaCl) + 3% Casein + 0.5% Tween-20) overnight, followed by affinity purified rabbit polyclonal antibodies directed against $GABA_{A\alpha2}$ (1:200; ProSci Inc., Poway, CA), $GABA_{A\alpha3}$ (1:2000; Sigma, St. Louis, MO), KCC2 (1:500, Millipore, Billerica, MA), and monoclonal anti-actin (1:3000, NeoMarkers, Fremont, CA) for 24 hrs. Following several washes with PBS, protein bands were detected using HRP-conjugated anti-rabbit secondary antibodies (1:1000, Jackson Lab., West Grove, PA) and HRP-conjugated anti mouse (1:3000, Thermo Scientific, Rockford, IL) and visualized using enhanced chemiluminescence detection. Signal intensities were quantified using Image j software (http://rsb.info.nih.go/ij).

Statistics: The chi-square distribution test was used to analyze distribution of GE cells within the neocortex. For all other analysis, a Student's t-test was applied and differences evaluated at $P < 0.05$.

Results

General properties of normal and MAM- treated migrating cells leaving the GE

We previously reported that cells leaving the GE in MAM-treated animals exhibit abnormal characteristics, specifically showing orientations that differed from control cells (Poluch et al., 2008). To further characterize the migratory behavior of cells leaving the GE or the CVZ, we applied an *in-vitro* live video-imaging assay to continuously view migrating cells. Our earlier work demonstrates that at P0-P1 in the ferret, the medial and lateral GE are fused, thus we will refer to our label as directed to the fused GE (Poluch et al. 2008). Migrating cells exiting the GE or CVZ of organotypic slices were labeled using electroporation with plasmids that code for RFP or injections of DiI. An example of a slice labeled by electroporation can be seen in Figure 1A. Higher power views of cells en route to the cortical plate are shown in Figures 1B and C. These cells have varied orientations as well as morphology; the dynamic features change when observed in real time. After allowing the cells to move away from the GE or CVZ, the dynamic pattern of migration was captured in real time. GE cells moving into the neocortex adopt different orientations and take different routes. A number of migrating neurons course through the VZ or SVZ (subventricular zone), many orient in a tangential direction (Figure 2A, B red arrows). A different group of more superficially labeled GE cells display both tangential (yellow arrows) and radial (green arrows) orientations. Many of the radially oriented cells point in the direction of the pial surface. However, some of

the radially-oriented cells have their leading edges directed toward the ventricular zone (Figure 2A, B, blue arrows). These observations correspond to our previous report in the ferret that similar to rodents, tangentially migrating cells in the GE follow 3 paths including one close to the VZ, one in the IZ, and one superficially near the marginal zone (not shown in this case) (Poluch et al. 2008). We also observed that migrating GE cells alter their morphology as they transit from point to point, generating distinct morphological features. In static images the labeled cells are either unipolar (the cell posses a single leading unbranched process (Figure 2C-a), display a branched process (Figure 2C-b), are bipolar (both leading and trailing processes Figure 2C-c), or multipolar (multiple processes emanate from the soma of the cell (Figure 2C-d).

Although the general trend of movement for cells leaving the GE is towards the neocortex, individual cells move in all directions and often alter their course of direction (Movie 1). Cells may emit one or more processes and move in the direction of the new branch (e.g. Movie 2), or emit branches and continue in the same direction or not move at all, but only explore the environment for a time (Movie 3). We also frequently observed nucleokinesis in which the nucleus moves within the cytoplasm of a cell in the direction of the leading process (as described by others such as Nadarajah et al. 2001, Martini et al. 2009, Movies 2 and 3). This process usually precedes the point-to-point translocation of the cell during migration.

Kinetic behavior of cells exiting the GE in normal and MAM-treated slices

Our earlier work indicates that after treatment with MAM, in addition to changes in orientation while migrating into the cortical plate, several subtypes of interneurons are redistributed in the neocortex of juvenile ferrets (Poluch et al. 2008). To assess the factors contributing to the altered distribution, we evaluated specific dynamic parameters of cells leaving the ganglionic eminence in normal and MAM-treated animals, including the speed, orientation of movement, and exploratory behavior (defined as extension of new processes and changes in the direction of movement).

Speed and direction. The migratory speed of GE and CVZ cells was measured as described in the Methods. Cells in organotypic cultures of normal ferrets migrated significantly faster (37.435 μm ± 0.87/hr) compared with similar cells in animals treated with MAM (32.98499 μm ± 0.73/hr) (Figure 3A). Because migrating interneurons from the GE have different orientations (e.g. Figure 2A and also Ang et al. 2003; Tanaka et al. 2003 and 2006), we examined the relationship of speed to orientation during movement and assessed distinctions between normal and MAM treatment for radial versus tangential travel. Normal cells traveling in both radial and tangential directions migrated significantly faster than MAM-treated cells (Figure 3B).

To investigate if changes in the speed of migration affect the final distribution of migrating neurons, we assessed their positions in organotypic slices of normal and MAM-treated animals after 48 hrs of incubation. We divided the neocortex into three roughly concentric regions (0-350 μm, 350-700 μm and greater than 700 μm) from the point of DiI injection in the GE using Axiovision

software (Figure 4A). The total number of migrating GE cells in each sector was computed; the amount of cells in each sector of the normal and MAM-treated organotypic slices were compared and presented as percentages of the total. Although slightly more cells in normal organotypic slices migrated further away from the GE as revealed by the higher percent in the region 700 μm from the GE, the overall distribution across the three sectors was not significantly different (Figure 4B). In other words, the cells leaving the MAM-treated GE moved slower, but eventually situate at similar distances away from the GE.

Exploratory behavior of GE-derived neurons in normal and MAM-treated slices

Migrating GE cells are influenced by a variety of signaling molecules that induce stereotypic migratory activity, which we refer to as exploratory behavior. Two parameters defined exploratory behavior in our study: (i) process extension or the ability of migrating cells to extend more than one leading process and (ii) turns or changes in direction of movement, in which a deviation from the initial course of movement occurred. In Figure 5A, the green arrow indicates a cell changing direction, while the red arrow shows a cell branching and also changing its direction of movement. To determine if treatment with MAM alters the ability of migrating GE cells to explore the neocortical environment, migrating cells were observed during live video imaging for 24 hrs. For several examples, the path of multiple migrating cells was traced using ImageJ. The movement was tracked for a period of at least 8 hours and the position of each cell recorded. Figure 6A and

B illustrate that the general pattern of movement in the MAM-treated tracks of cells (Figure 6B) show fewer changes in their direction when compared with the normal patterns of movement (Figure 6A). The quantification of movement patterns is seen in Figure 5B, which demonstrates that the tracks of normal cells completed more changes of direction in their movement patterns (i.e., turns) compared with the MAM-treated cells. The percent of cells displaying process extension or turning in each slice was calculated for normal and MAM-treated organotypic cultures. Cells were scored negative if they did not display any of these behaviors during the observed period of time. In MAM-treated slices, fewer cells leaving the GE extended more than one leading process in comparison with cells in normal animals. In addition, the percent of cells that performed turns was fewer after MAM treatment (Figure 5B).

Interference with $GABA_A$-mediated activity affects migratory behavior of GE cells in MAM-treated animals

The migratory behavior of interneurons entering the neocortex is regulated by a variety of signaling molecules including GABA (Cuzon et al. 2006; Heck et al. 2007; Lopez-Bendito et al. 2003 and 2008). We previously demonstrated that treatment with MAM leads to a widespread redistribution of $GABA_{A\alpha}$ receptors; we also found that increasing $GABA_{AR}$-mediated activity in normal organotypic cultures interferes with the orientation of interneurons as they migrate into the cortical plate similar to the effect of MAM treatment (Jablonska et al. 2004; Poluch et al. 2008). These findings suggest that sensitivity to GABA is unusually

high after MAM treatment and inhibiting GABA signaling may alter the positioning of neocortical interneurons. We predicted that treatment with $GABA_A$ antagonists would shift the kinetic behavior of neurons originating from MAM-treated slices toward normal. The speed and exploratory behavior of migrating cells in normal and MAM-treated slices was evaluated as described previously after adding the $GABA_A$ antagonist BMI to the media. This treatment significantly increased the speed of migration in the MAM-treated group to be closer to normal (Figure 7A).

When organotypic slices of E33 MAM-treated animals were exposed to BMI, the percent of migrating cells extending more than one leading process increased and was nearly identical to those of normal migrating GE cells. This matches our prediction that decreasing $GABA_A$ activity in MAM-treated brains results in more normal behavior (Figure 7B). The number of turns, or alterations in the direction of movement, did not change however, after adding BMI to the media.

Treatment with MAM does not significantly alter the migration kinetics of neurons originating from the cortical ventricular zone

Our previous observations indicated that tangentially migrating GABAergic neurons were specifically disoriented in MAM-treated ferrets (Poluch et al. 2008). To test if the changes in the kinetic behavior of migrating interneurons were limited to the neurons originating from the GE, we examined the migration of neurons from the CVZ to the neocortex using live-video imaging. Organotypic slices were injected with DiI in the neocortical ventricular zone and only slices in

which the DiI label was confined to the VZ were used for analysis. In this regard we limited labeling cells in the vicinity of the CVZ, but arising from the GE. We hoped to insure that the cells migrating away originated from the CVZ. An example of an injection site and cells migrating away from the CVZ can be seen in Figure 8. We measured the speed and exploratory behavior of cells leaving the CVZ in normal and MAM-treated slices. Although we found no significant differences in the parameters analyzed between the two groups, there was a noticeable, but statistically insignificant, decrease in the speed of migration for the MAM-treated cells (Figure 9A-B).

Treatment with MAM increases the expression of $GABA_{A\alpha2}$, $GABA_{A\alpha3}$ receptor subunits and KCC2

Our previous studies strongly suggest that $GABA_A$ receptors and GABA activity are increased in the neocortex of MAM-treated animals (Jablonska et al. 2004; Poluch et al. 2008). Here we also show that blockade of $GABA_A$-mediated activity ameliorates the deficits in dynamic activity after MAM treatment. To further assess the role of GABA in mediating the kinetics of migrating GE cells following treatment with MAM, we determined the level of expression of $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ receptor subunits using Western blots. $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ show high expression in both the neocortex and GE early in development, and may play important roles in migration and other developmental events in GE cells (Laurie et al. 1992; Poulter et al. 1992; Fritschy et al. 1994; Wang and Kriegstein 2009; Carlson et al. 2010). In our study, both α2 and α3 subunits are increased in

MAM- treated cortex compared to normal, furthering support for increased $GABA_A$-mediated activity in our model animals (Figure 10A and B).

To further evaluate the potential of altered environmental GABA to influence migration, we established the amount of KCC2 in normal and MAM-treated cortex. During development, KCC2 maintains a high extracellular Cl^- gradient and induces hyperpolarization of cells in response to GABA, playing a role in the developmental switch from the GABA-induced depolarization to hyperpolarization, which may also terminate cell migration (Rivera et al 1999; Bortone and Polleux 2009). After MAM treatment, both monomeric and oligomeric forms of KCC2 were increased in the neocortex as demonstrated using Western blots (Figure 10C, right upper panel). Furthermore, ratiometric analysis reveals a decrease in KCC2 monomer: oligomer ratio (Figure 10C, right lower panel).

Discussion

The kinetic behavior of migrating interneurons, influenced by both intrinsic and environmental factors, plays an important role in the proper positioning of these cells in the neocortex. We developed a model of cortical dysplasia in ferrets by interrupting the development of layer 4 cells resulting in abnormal placement of specific populations of interneurons (Noctor et al. 1999; Poluch et al. 2008). In this study we show that transient and layer-specific interference with corticogenesis leads to: a) alterations in the basal activity of $GABA_A$ and in KCC2 expression b) this change directly influences the kinetic behavior of migrating GE cells. These findings directly and uniquely implicate increased $GABA_{AR}$ and KCC2 activity in impairing dynamic aspects of migration and in the ability of GE cells to actively explore the environment during migration to the cerebral cortex. We demonstrate that details of migration not shown before involving active exploration are strongly influence by ambient $GABA_{AR}$ and KCC2 activity. This may underlie the modified migratory behavior of later born interneurons.

Ferrets signify a valuable developmental model. As the smallest mammal with a convoluted cortex, they represent an important link between studying lissencephalic rodents and non-human primates. Several groups have identified important developmental features in ferrets, including our previous findings that many neurons populating the neocortex are generated in lateral portions of the GE (Poluch et al. 2008). Several recent articles describe a distinct outer subventricular zone (oSVZ) in ferrets, a region found in primates, and suggest that this region may be important for generating cells that populate and expand

the gyrencephalic neocortex (Fietz et al. 2010; Reillo and Borell 2011; Reillo et al. 2011; Liu et al. 2011). The oSVZ contains a population of cells called outer radial glia (oRG; also called tRG), which appear to be the cell type responsible for generating additional cortical cells leading to the expansion of the developing cortex. This idea is contested recently by a group suggesting that the outer SVZ in ferrets does not differ substantially from that region in the rat (Martinez-Cerdeno et al. 2012). Nevertheless, these examples demonstrate the importance of studying developmental processes using several animal models.

MAM treatment alters the kinetic behavior of migrating GE cells

The ability of migrating GE cells to reach appropriate positions within the neocortex depends on the concerted action of extrinsic factors within the neocortex and intrinsic signaling mechanisms that inhibit, facilitate, and guide migrating neurons (Anderso et al. 1997; Powell et al. 2001; Lopez-Bendito et al. 2003 and 2008; Polleux et al. 2003; Stumm et al. 2003; Alifragis et al. 2004; Flames et al. 2004; Cuzon et al. 2006; Cobos et al. 2007; Liodis et al. 2007; Friocourt et al. 2008). Events leading to changes in neocortical architecture may cause variability in the environment that alters the pattern of migration of neuronal cells and results in abnormal cell positioning and cortical dysplasia. In our model of cortical dysplasia, layer 4 of ferret neocortex is diminished resulting in widespread termination of thalamocortical afferents (Palmer et al. 2004), redistribution of $GABA_A$ receptors and parvalbumin and calbindin positive interneurons (Jablonska et al. 2004, Poluch et al. 2008) and changes in the

sequence of information transfer in the somatosensory cortex (McLaughlin et al. 2005). To better understand the mechanisms contributing to the redistribution of interneurons, we evaluated several parameters of kinetic behavior in migrating GE cells. Cells leaving the GE in normal animals migrated significantly faster compared to similar cells in MAM-treated animals. In addition, GE generated neurons in MAM-treated animals exhibited less exploration of the neocortical environment. Exploratory behavior, including extension of multiple leading processes and changes in direction of movement, results from coordinated signaling from various sites, which communicate cues that determine the final location of these cells. The change in the speed of migration and exploratory activity of interneurons following treatment with MAM therefore has important implications on the overall positioning of cells within the neocortex.

Inhibiting $GABA_A$-mediated activity improves migration of interneurons in MAM-treated animals

GABA is an important influence on migrating cells, although its precise role in the migration of telencephalic cells is complex. GABA receptors are important during different phases of migration and mediate aspects of the initiation, continuation, and termination of cellular movement (Behar et al. 1996, 2001; Bolteus and Bordey 2004; Heck et al. 2007; Bortone and Polleux 2009). Cuzon and colleagues (2006) report that $GABA_A$ activity is present throughout the path of migration of mouse GE cells into the neocortex, but increases in activity as neurons approach and enter their cortical target. Our studies suggest

that MAM treatment results in increased ambient $GABA_A$-mediated activity, which may interfere with migration of GE cells as they move en route to the neocortex before the final phase identified by Cuzon et al. 2006. This is supported by our original observation of expanded $GABA_{A\alpha}$ immunoreactivity in MAM-treated cortex (Jablonska et al. 2004) and the current finding of increased $GABA_{A\alpha 2}$ and $GABA_{A\alpha 3}$ receptor subunits as demonstrated by Western blot. Both α2 and α3 subunits of the $GABA_A$ receptor are expressed early during development and may mediate the initial roles of GABA during development (Laurie et al. 1992; Poulter et al. 1992; Fritschy et al. 1994; Carlson et al. 2010). This assists in explaining our finding that elevated levels of GABA interfere with migration of GE cells to slow the pace but not prevent their movement.

To investigate the involvement of $GABA_A$-mediated transmission in the migration of interneurons, we manipulated the level of $GABA_A$ activity in MAM-treated organotypic cultures using BMI to reduce the ambient $GABA_A$ activity. We predicted that limiting the increased $GABA_A$ activity would change the cellular actions in MAM-treated cortex to be more like normal. In fact, blocking $GABA_A$ activity in MAM-treated animals resulted in significant enhancement of the migration speed of cells leaving the GE as well as increasing the percentage of MAM-treated GE cells extending more than one leading process. This suggests that reducing the effectiveness of ambient GABA in the MAM-treated ferret encourages migratory activity to become more normal.

Increased GABA signaling interferes with migration

Both $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ expression are increased in MAM-treated animals compared to controls. Does increased $GABA_{A_\alpha}$ expression in MAM-treated animals alter the kinetic behavior of GE cell migration? It has been known for several years that GABA acts as an excitatory neurotransmitter early in development to facilitate cell migration, but switches to mediate inhibition as cells mature (Plotkin et al. 1997; Clayton et al. 1998; Rivera et al. 1999). The dual role of GABA is attributed to the temporal expression of two cation-chloride transporters, sodium-potassium-chloride (NKCC1) and KCC2. Migrating neurons initially express NKCC1, which imports chloride ions (Cl^-) into the cell to maintain a higher intracellular Cl^- concentration (Clayton et al. 1998). In response to $GABA_A$ activation, the outward movement of Cl^- results in depolarization of the cells and triggers Ca^{2+}-mediated events that influence the cytoskeleton and stimulate migration (Behar et al. 2001; Soria & Valdeolmillos 2002). As cells mature, NKCC1 downregulates and coincides with increased expression of KCC2 (Plotkin et al. 1997). KCC2 is a Cl^- exporter that maintains a higher extracellular gradient of Cl^-. Consequently, $GABA_A$ activation when KCC2 is increased results in hyperpolarization and cell inhibition (Payne, 1997; Rivera et al. 1999). Interestingly, in dissociated hippocampal cell cultures, activation of $GABA_{AR}$ induces the expression of NKCC1 and KCC2 (Ganguly et al. 2001). In addition, pharmacologic blockade of $GABA_A$ delayed the expression of KCC2, while increased activity of $GABA_{AR}$ induces precocious expression of the transporter. Premature expression of KCC2 as a result of a heightened state of $GABA_A$–mediated activity, as we see here, may be responsible for the migration

defects observed in MAM-treated ferrets. KCC2 is implicated in several important processes, including decreased motility as a neuron migrates, which leads to cell slowing, stopping, and differentiating. As a consequence, a premature switch of the $GABA_A$ response may occur and produce early arrest of migrating interneurons. Bortone and Polleux 2009, demonstrated that depolarization through $GABA_A$ receptors stimulates motility of GABAergic migrating neurons while hyperpolarization of $GABA_A$ receptors, induced by KCC2 expression, provides a stop signal for migrating cells. We observed increased expression of both KCC2 monomer and oligomers in the neocortex of young MAM-treated animals. The ratio of KCC2 monomer to oligomer was also reduced in MAM-treated animals, which indicates greater activity of this transporter in our model animals. Oligomerization of KCC2 is an important step in the activation and function of KCC2 as a Cl^- exporter (Blaesse et al. 2006). Although others have demonstrated that $GABA_{AR}$ and KCC2 activity are involved in neuronal migration, we demonstrate here that high endogenous levels of these substances directly interfere with migration. In addition they appear specifically important in dynamic and exploratory behavior in cells leaving the ganglionic eminence.

MAM treatment has no effect on the kinetic profile of migrating cortical VZ cells

To determine whether the effect of MAM treatment on the kinetics of migration is restricted to interneurons alone, we also investigated the kinetics of migrating neurons arising from the cortical VZ. Although the migration speed of

CVZ cells was reduced in MAM-treated animals, the deficit was not significant. We also did not observe any significant differences in exploratory behavior between cells in normal animals and those that received MAM treatment, indicating the impact might be restricted to tangentially migrating interneurons. The differential impact of MAM-treatment on the kinetic behavior of GE and CVZ-derived cells relative to normal cells is not surprising since these two cell populations have different neurogenic origin and migrate under the influence of similar but not identical signaling mechanisms (Marin and Rubenstein, 2003).

A working model. A plethora of signaling molecules influence migrating interneurons. GABA initially acts as a stimulating signal to facilitate migration and then acts as a stop signal to arrest the migratory process, an effect that correlates with the sequential expression of NKCC1 and KCC2. An appropriate level of GABA is necessary for the proper migration and positioning of interneurons. Our data suggests that following treatment with MAM, the basal level of $GABA_A$ receptor ($GABA_{AR}$) activity increases, and the elevated level of $GABA_{AR}$ activity impairs the migration of interneurons, through premature expression of KCC2. Thus, treatments that decrease the level of $GABA_{AR}$ activity enhance the migration of interneurons in part by slowing the development of KCC2.

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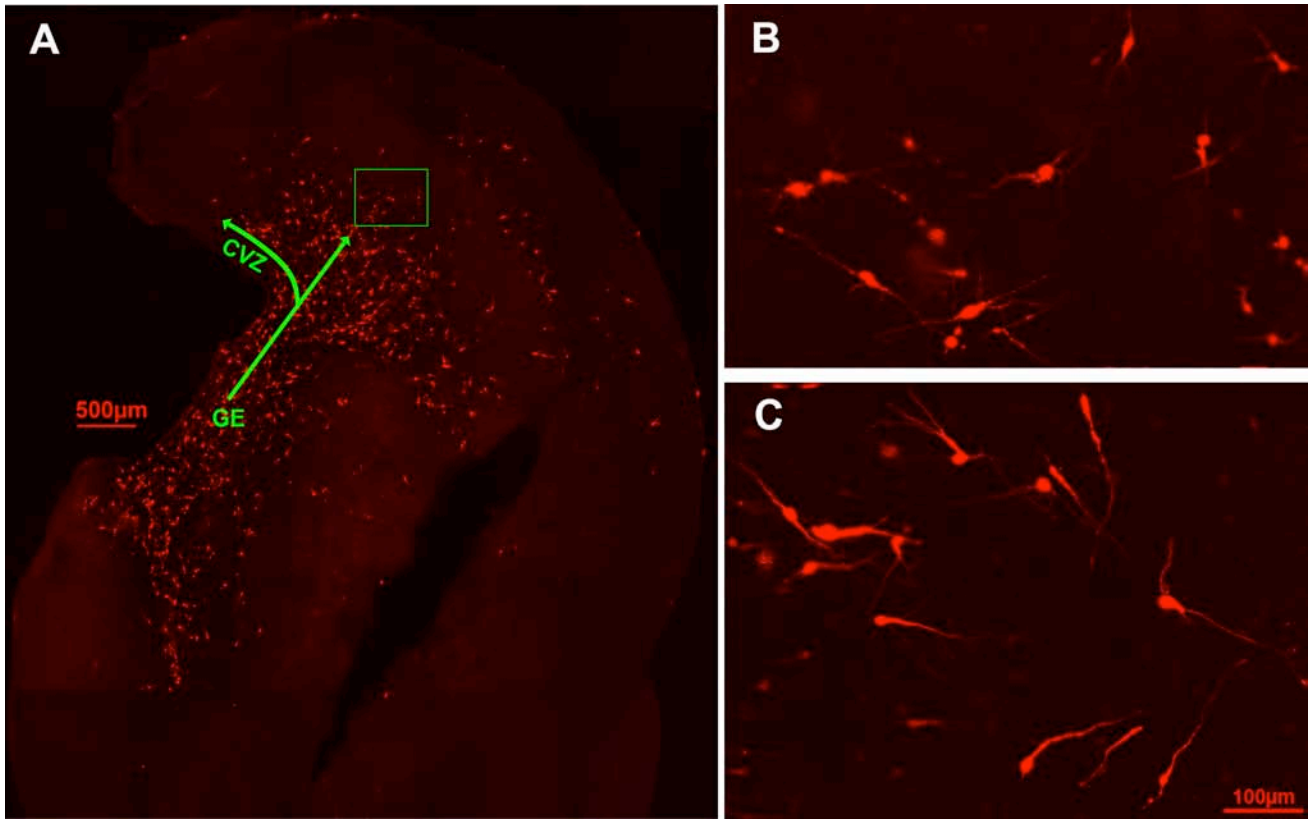


Figure 1. Images of migrating neurons leaving the ganglionic eminence (GE) of a normal ferret. (A) Example of an organotypic culture obtained from a P0 ferret labeled by electroporation using a plasmid that codes for red fluorescent protein (RFP). (B-C) Higher power views of cells en route to the cortex revealing varied orientations and morphologies of migrating GE cells. B is taken from the boxed in region of A and C is from a different normal slice culture. The arrows indicate the path of the migrating cells. GE: ganglionic eminence; CVZ: Cortical ventricular zone

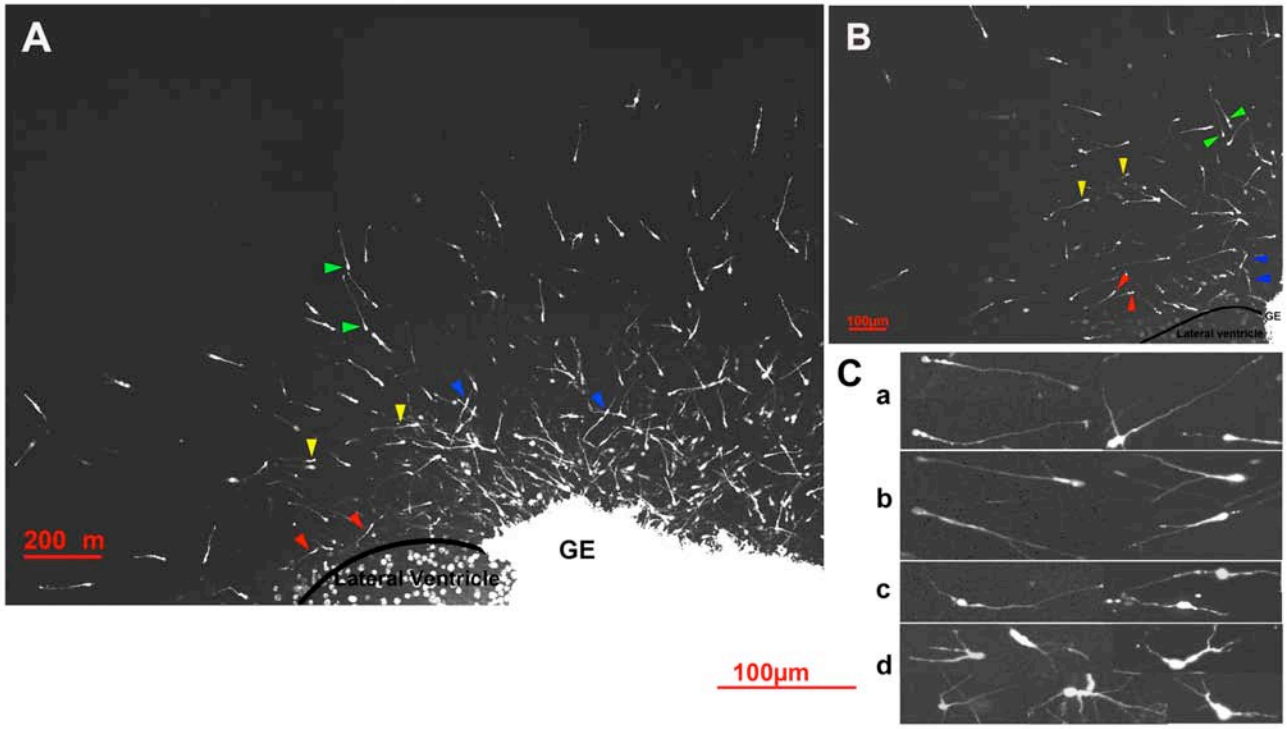


Figure 2. Migrating GE-derived neurons labeled with DiI. (A) Neurons migrating from the GE in an organotypic culture of a MAM-treated animal display different orientations and positions within the neocortex: tangentially migrating GE cells within the VZ/SVZ region (red arrows), tangentially migrating GE cells within the intermediate zone (yellow arrows), radially-oriented GE cells in the intermediate zone (green arrows) and radially-oriented GE cell directed towards the ventricle (blue arrow). (B) A different organotypic culture of a MAM-treated animal labeled with DiI, showing similar orientations of migrating cells. Arrows mark the different orientations and positions of cells within the neocortex as described above. (C) Higher power images of cells obtained from several labeled organotypic cultures from both normal and MAM-treated animals (the images on the left and right were taken from normal slices); various morphologies can be seen including those with a single leading unbranched process (a), those with single leading but branched processes (b), bipolar cells (c), and cells with multiple leading processes (d). GE: ganglionic eminence; LV: Lateral ventricle

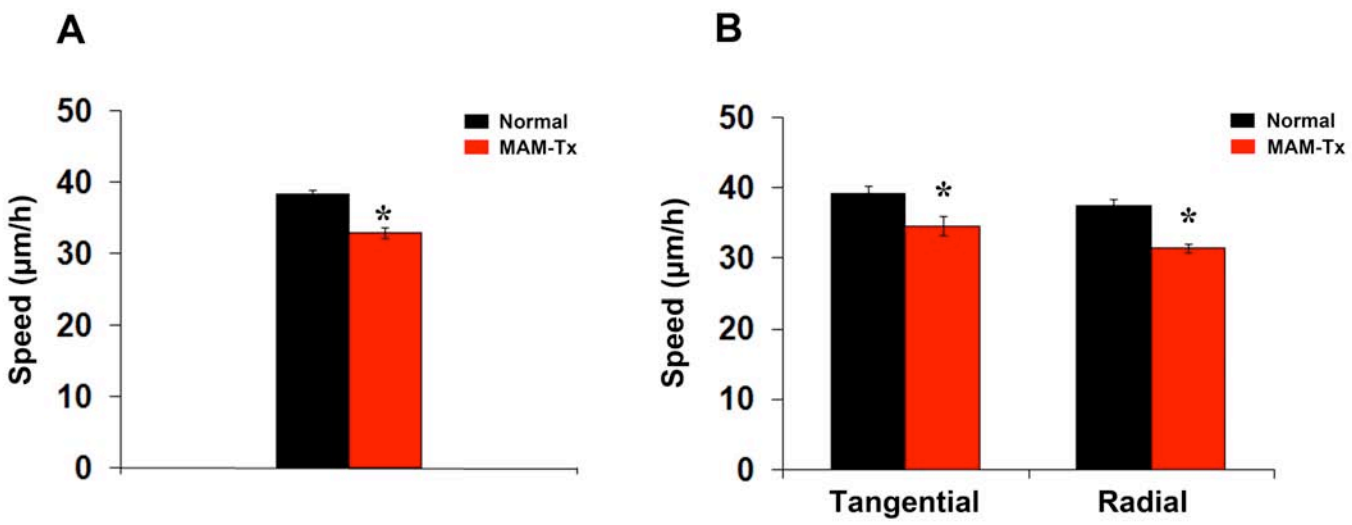


Figure 3. Effect of MAM treatment on the speed and orientation of migrating interneurons leaving the ganglionic eminence and traveling to the neocortex. (A) Neurons leaving the GE travel faster in normal slices compared with those in MAM-treated slices, *n*= 390 cells (normal) and 467 cells (MAM-treated). (B) Cells traveling in either the radial or tangential direction travel slower in MAM treated slices. Tangentially migrating cells: *n* = 129 (normal), 105 (MAM-treated); radially migrating cells: *n* = 223 (normal), 348 (MAM-treated). Significance determined using a Student's t-test; * $p < 0.05$. Error bars = Standard error of mean.

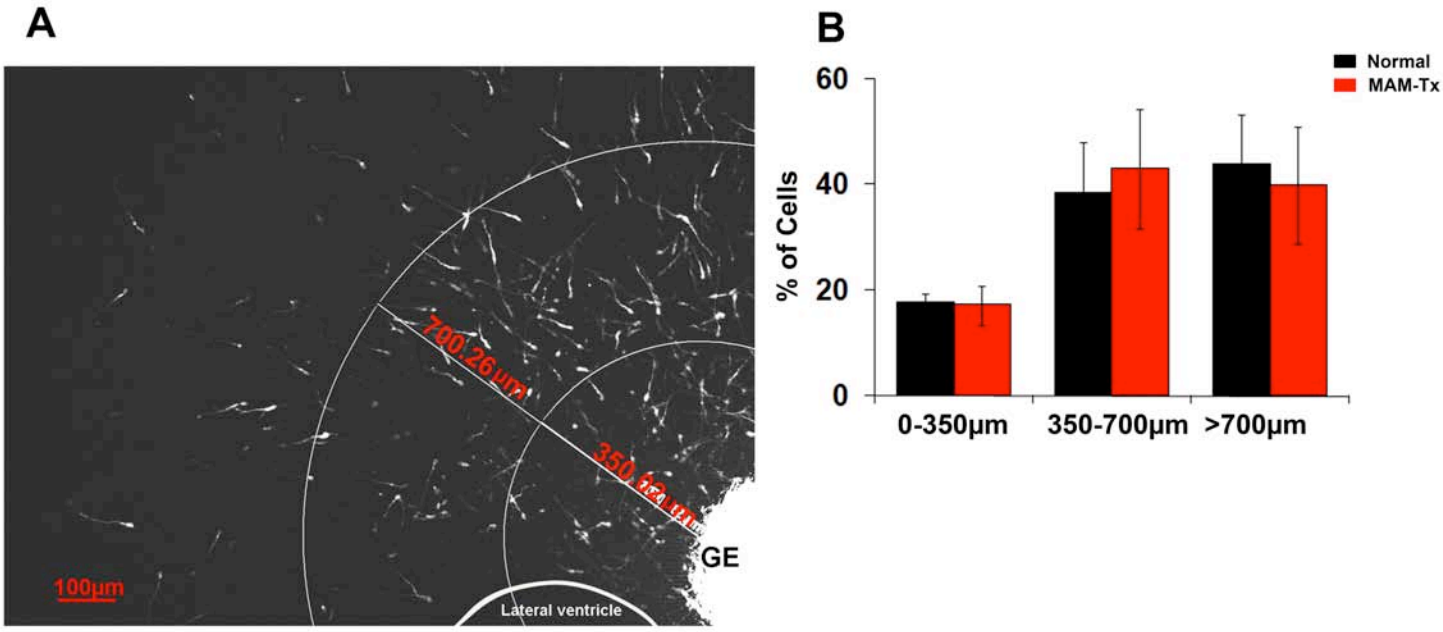


Figure 4. Distribution of cells leaving the GE and traveling to the neocortex. (A) To determine if cells migrated different distances after treatment with MAM roughly concentric regions were created in order to count the neurons that traveled up to 350 µm from the border of the GE VZ, from 350 to 700 µm from that border, or greater than 700 µm from the center of DiI injection. (B) After 48 hrs in culture there were no significant differences in the overall distribution of cells leaving the GE in normal slices vs those in MAM- treated slices, A Chi-Square distribution showed no significant differences. *n=12* slices (MAM-treated), *10* slices (normal). Error bars = Standard error of mean. GE: ganglionic eminence.

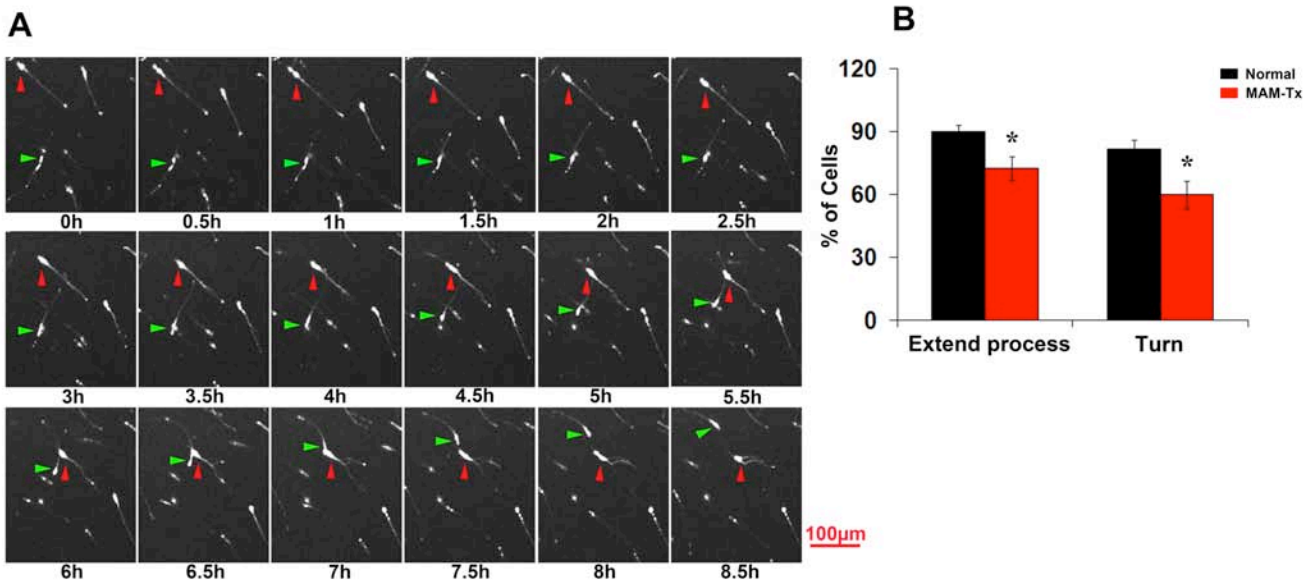


Figure 5. Effect of MAM treatment on the ability of migrating GE cells to extend multiple leading processes and initiate a change in direction of movement. (A) Sequence of images of DiI-labeled GE cells during time-lapse imaging for 8.5 h. Two cells are indicated with either a red or green arrow and followed over time. The cell labeled with the red arrow moves diagonally from the upper left to the approximate center of the image. At 6.5 h it branches and moves toward the right. The cell labeled with a green arrow remains relatively still from 0 -. 4h. At this point it begins to move upward and curves to the left, moving out of view at 8.5h. (B) The percent of migrating cells extending new processes was evaluated in normal and MAM-treated slices. After MAM treatment, migrating cells extended fewer new processes and displayed fewer turns/changes in the direction of movement. *n=23* slices/366 cells (MAM-treated), *21* slices/239 cells (normal); *$P \leq 0.05$, Student's t-Test. Error bars = Standard error of mean.

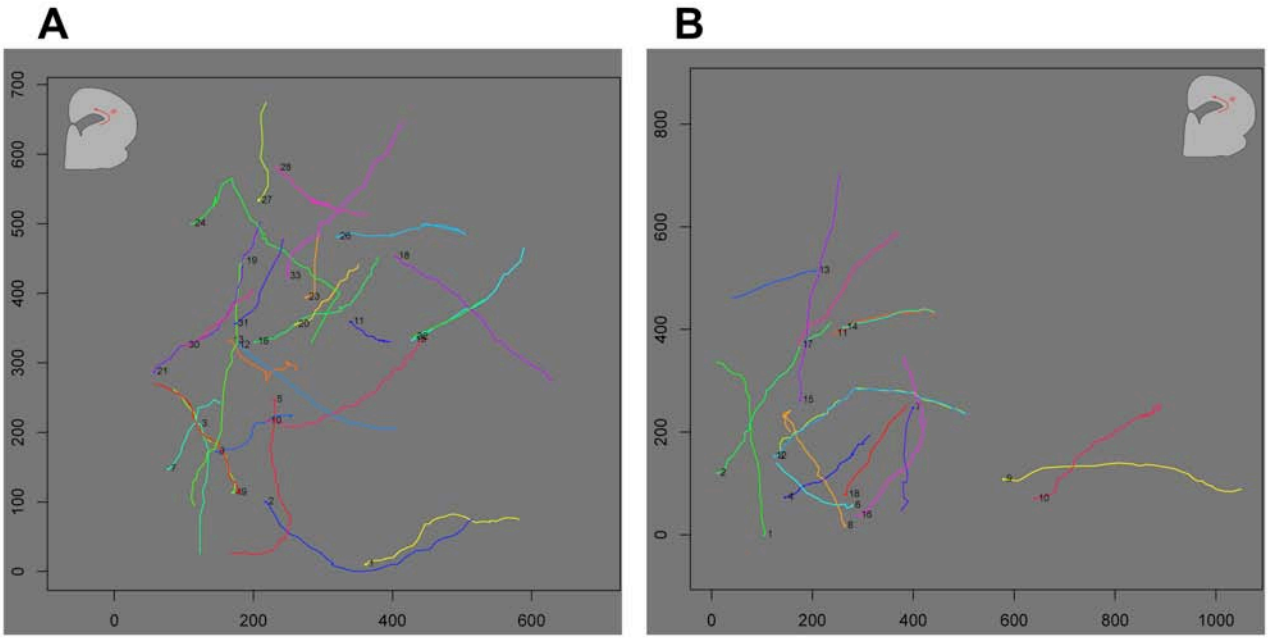
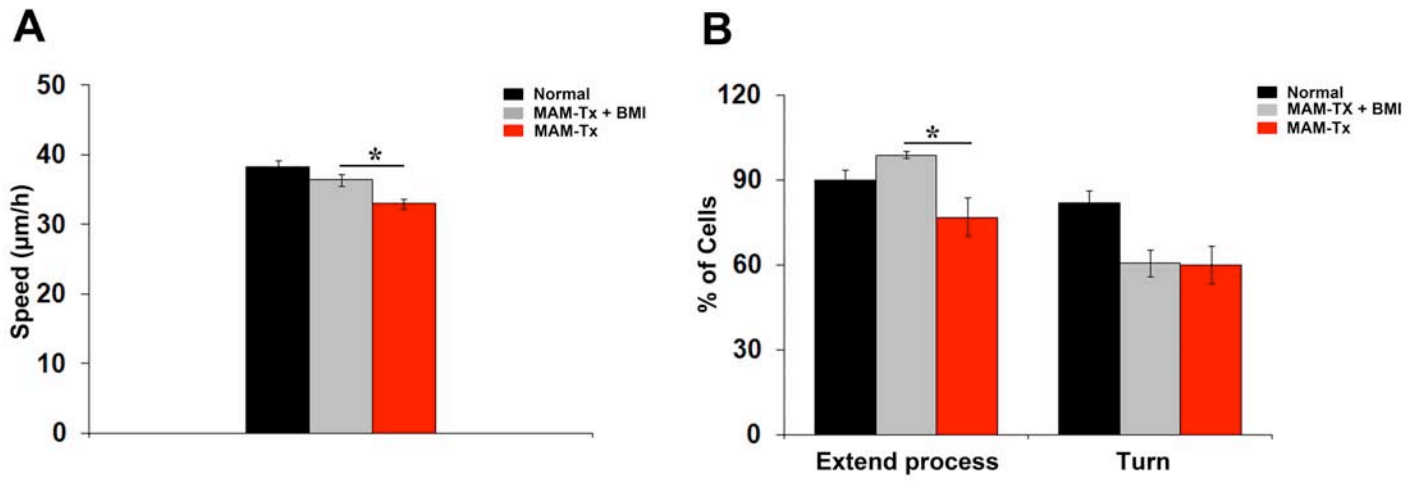


Figure 6. Tracks of cells in normal and MAM treated slices. (A) The positions of migrating cells were tracked every 30 minutes over a period of 24 h. Each cell is indicated with a different color and a number. This is an example taken from a normal cortex. (B) The tracks of cells leaving the GE of MAM-treated cortex are indicated here. The position of each cell is illustrated with a different color and number. The cells leaving the normal GE show more variability in their route, compared with those leaving the GE of MAM-treated brains. The numbers on the x and y axis indicate the size of the area of the neocortex analyzed while the length of each track indicates a rough estimate of the distance traveled in micrometers. The outline of ferret cortex in A and B indicates the approximate path of cell movement (red arrow).



F**igure 7**. Administration of bicuculline (BMI) to MAM-treated slices results in an increase in the speed and exploratory behavior of migrating cells. MAM-treated slices were placed in culture with BMI added to the medium. (A) The addition of BMI increased the speed of migration in the treated slices. (B) The addition of BMI also improved the ability of MAM-treated cells to extend processes. The number of turns in the direction of movement did not increase. Significant differences are only shown between the 2 conditions of MAM treated slices, i.e. MAM-Tx alone (red bars) or MAM-Tx with BMI added (gray bars). The normal condition is shown for reference (black bars). BMI treatment also eliminated significant differences between normal and MAM-Tx for speed and extension of processes. Speed: *n* = 467 cells (MAM-treated), 270 cells (MAM-treated + BMI); exploratory behavior: *n* = *23* slices/366 cells (MAM-treated), *16* slices/107 cells (MAM-treated +BMI), * $P \leq 0.05$, Student's t-Test. Error bars = Standard error of mean.

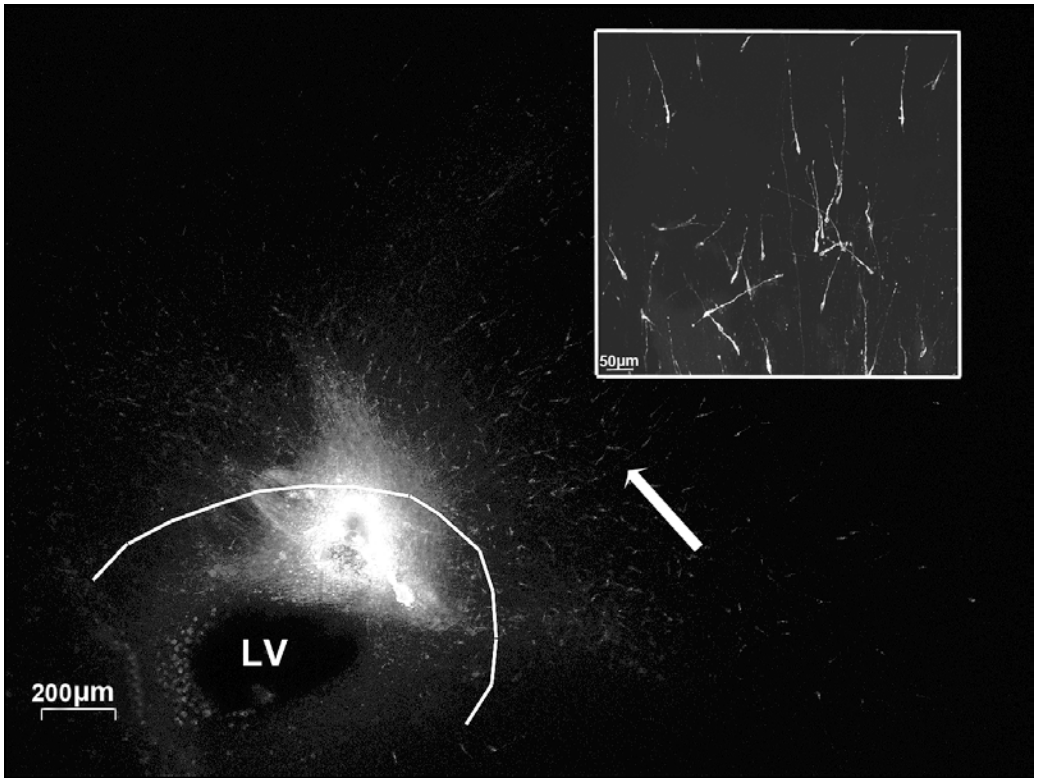


Figure 8. Example of an injection of DiI into the neocortical ventricular zone of an organotypic culture obtained from a MAM-treated animal. The ventricular zone is outlined with a white line. Labeled neurons can be seen migrating toward the neocortex. The inset shows labeled migrating cells from the same image moving into the cortex. The image in the inset was taken from the region indicated with an arrow. LV, lateral ventricle.

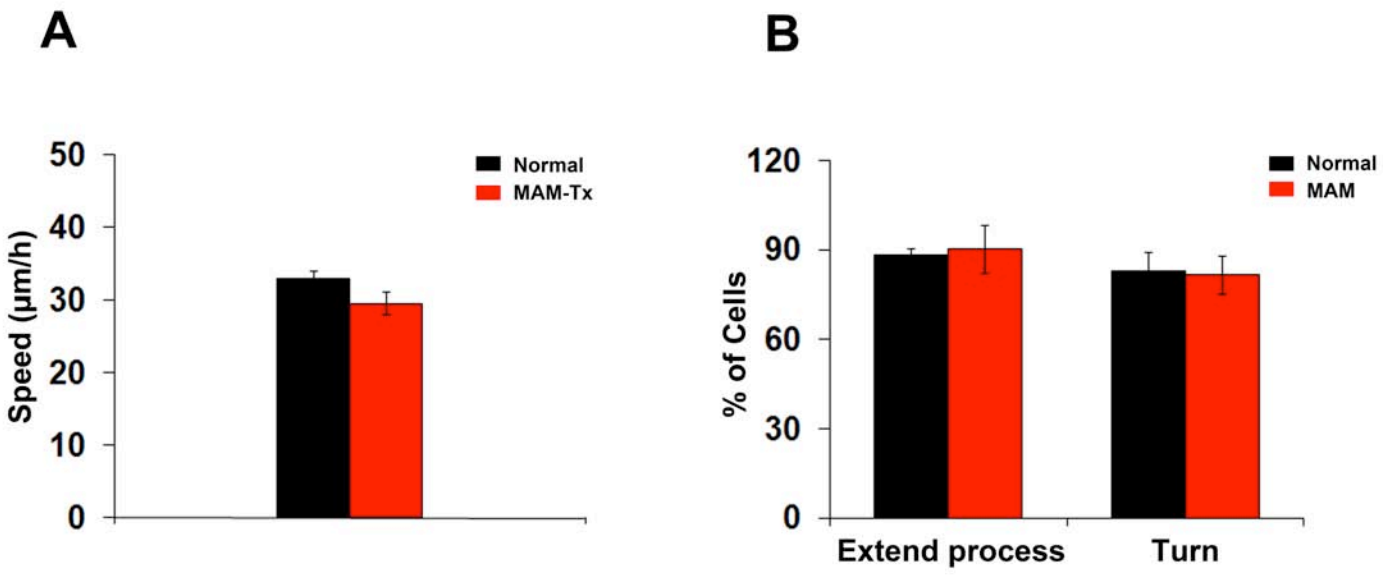


Figure 9. Effect of MAM treatment on the speed of migration and exploratory behavior of cells leaving the cortical ventricular zone (CVZ) to the neocortex. (A) There were no significant differences in the speed of migration of cells leaving the CVZ from either normal or MAM-treated slices *n*= 191 cells (normal) and 185 cells (E33 MAM), or (B) in the ability of cells to extend processes or change the direction of movement or turn. Exploratory behavior: n= 13 slices/85 cells (normal) and n= 12 slices/75 cells (MAM-treated).

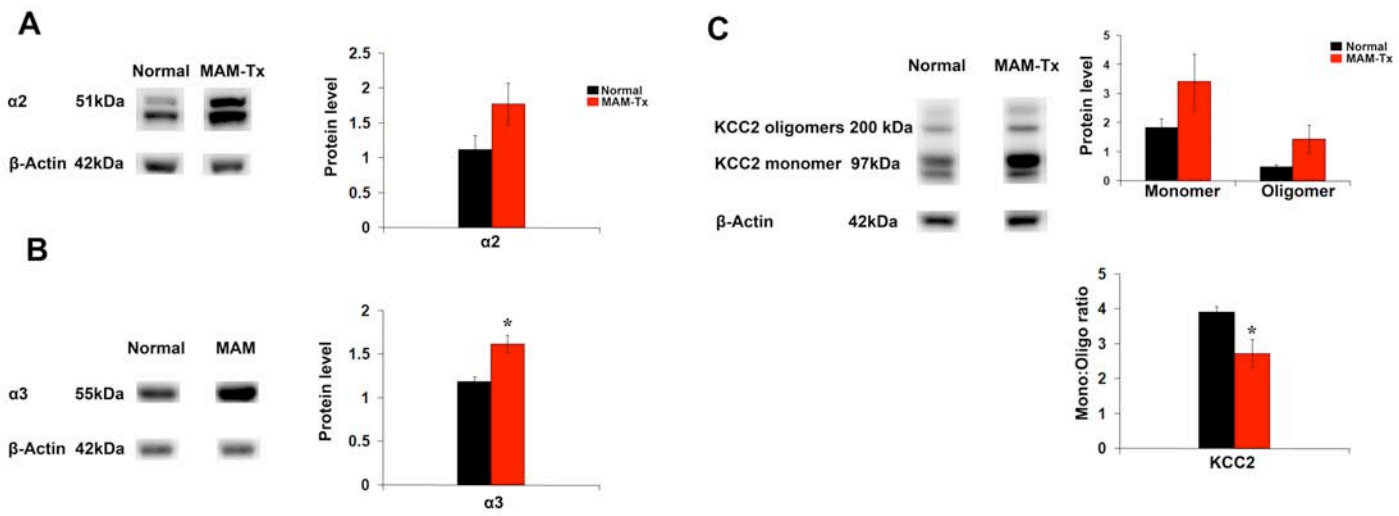


Figure 10. Expression of $GABA_A$ receptor subunits and KCC2 following treatment with MAM. (A) Treatment with MAM results in an increase in the expression of $GABA_{A\alpha2}$, and (B) $GABA_{A\alpha3}$ receptor subunits in the neocortex as demonstrated by Western blots. (C) Both monomeric and oligomeric forms of KCC2 are increased (top bar graph) with significant elevation in the ratio of KCC2 oligomer:monomer (bottom graph). Protein levels were determined by densitometric analysis using ImageJ software. For both normal and MAM-Tx animals, n = 4. Significance determined using the Student's t-test, * $P< 0.05$. Error bars = Standard error of mean.

Movie 1. View of cells leaving the ganglionic eminence en route to the cerebral cortex. This movie shows cells leaving the ganglionic eminence that move towards the cerebral cortex, which is located in the upper left of the images. It took place over a period of 16 hours, images were obtained every 30 minutes. Although the overall flow of movement was toward the neocortex, many cells make turns on their way or travel in opposite directions, toward the ventricular zone or in more lateral directions. Cells were labeled with DiI. This is taken from a MAM-treated cortical slice at P0.

Movie 2. Cells exiting the ganglionic eminence migrating to the cerebral cortex. There are fewer cells in this image, which allows easier viewing of details. Individual cells stop and explore the environment without obvious movement. Other cells change direction of movement or extend processes. Nucleokinesis occurs in many cells. This takes place over a period of 16 hours, with images obtained every 30 minutes. This is taken from a MAM-treated slice at P0.

Movie 3. Single cell branching and exploring. This movie shows a single cell that exited the ganglionic eminence and moves toward the cerebral cortex. The cell initially shifts ventrally and then changes direction toward the cortex. It also appears to explore the environment by extending processes. This movie takes place over a period of 9 hours, with sampling every 30 minutes. It is taken from a normal slice at P0.

CHAPTER 3

Targeted disruption of layer 4 formation increases $GABA_A$ receptor-mediated activity in upper cortical layers.

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Abstract

Cortical dysplasia is frequently associated with various clinical pathologies including epilepsy and mental retardation. This disorder results from impaired migration of immature neurons to their cortical targets, leading to anomalous clustering of neural cells and changes in cortical properties. We developed a model of cortical dysplasia by administering methylazoxymethanol (MAM), an anti-mitotic, to pregnant ferrets on embryonic day 33 (E33) to disrupt the birth of layer 4 cells leading to a significant reduction in the thickness of this layer and a redistribution and increased expression of $GABA_A$ receptors. We evaluated the impact of MAM-treatment on $GABA_A$ receptor-mediated synaptic transmission in both migrating neurons derived from the ganglionic eminence (GE) in organotypic cultures and on pyramidal cells in layer 2/3 of acute slices of postnatal day 28-38 (P28-P38), using whole-cell patch-clamp recording. E33 MAM treatment results in a significant increase in the amplitude and frequency of spontaneous $GABA_A$ receptor-mediated inhibitory post-synaptic currents (IPSCs) in migrating GE cells. In older MAM-treated animals, the amplitude of $GABA_A$ receptor-mediated spontaneous IPSCs in layer 2/3 pyramidal cells increased with no changes in frequency. The amplitude and frequency of miniature IPSCs, however, were significantly increased as a result of MAM-treatment in the older pyramidal cells. The kinetics of $GABA_A$ receptor opening also altered following treatment with MAM. Western blot analysis shows that the expression of $GABA_{A\alpha3}$ receptor subunit increased in our model animals. Taken together, these observations reinforce the idea that synaptic neurotransmission through $GABA_{AR}$

is enhanced following treatment with MAM and coincides with our previous finding of increased expression of $GABA_{A\alpha}$ receptor expression within the upper cortical layers. Overall, we demonstrate that small amounts of toxins delivered during corticogenesis can result in long lasting changes in the neural architecture that manifest as subtle disorders of information processing.

Key words: *cortical dysplasia, GABA, patch-clamp, western blotting*.

Introduction

During development of the mammalian neocortex, immature neurons generated in the germinal zones of the neocortex and ganglionic eminences (GE) migrate to create the six-layered, inside-out model of the neocortex (Angevine and Sidman 1961; Rakic 1974). This laminar organization is crucial for establishing a balance between excitation and inhibition leading to the overall function of the neocortex. Events with adverse impact on neuronal migration lead to cortical dysplasia (CD), a developmental abnormality characterized by aberrant distribution and clustering of neocortical cells resulting in a plethora of neuropathological disorders such as epilepsy, schizophrenia, and mental retardation (Taylor et al. 1971; Choi and Mathias 1987; Palmini et al. 1991; Gleeson and Walsh 2000; Zhu and Roper 2000; Ross and Walsh 2001; Tassi et al. 2002; Calcagnotto et al. 2002; Calcagnotto and Baraban 2003; Colombo et al. 2003; Guerrini et al. 2003; Moroni et al. 2005, 2008). A common pathological finding in clinical cases and animal models of CD is anomalous distribution and clustering of neurons within the neocortex, which alters the microcircuitry and cortical architecture (Ferrer 1993; Roper 1998; Colacitti et al. 1999; Jacobs et al. 1999; Benardete and Kriegstein, 2002; Moroni et al. 2008). Importantly, abnormal GABA signaling frequently associates with cortical dysplasia leading to consequent alteration in the balance of excitation and inhibition and disruption of normal cortical function (Roper et al. 1999; Zhu and Roper 2000; Benardete and Kriegstein 2002; Xiang et al. 2006; Moroni et al. 2008; Zhou and Roper 2009; Brill and Huguenard 2010; Zhou and Roper 2010, 2011).

We developed a model of cortical dysplasia in ferrets using methylazoxymethanol (MAM). We took advantage of the pharmacokinetic property of MAM as a short-acting anti-mitotic, and the long developmental time course of ferret corticogenesis to selectively disrupt the birth of layer 4 by delivery on embryonic day 33 (E33) (Noctor et al. 1997; Noctor et al. 1999; Palmer et al. 2001). The ferret, as the smallest mammal with a gyrencephalic cortex, is crucial for the study of neocortical development. Because ferrets possess an expanded neocortex with sulci, gyri, and large amounts of white matter it is distinctive as a research subject. The protracted period of corticogenesis and long period of cell proliferation in the large outer subventricular zone may contribute to the expanded cerebral cortex in the ferret compared with the rodent (Fietz et al. 2010). Distinctive features in the ferret related to neocortical development make it essential to study developmental processes in this animal, since aspects of neurodevelopment differ in mammals with a convoluted versus lissencephalic cortex (Kriegstein et al. 2006; Poluch et al. 2008).

As a likely result of the loss of layer 4 in our model, thalamic afferents that normally synapse directly in this layer become redistributed to upper and lower cortical layers; the capacity for entrainment and information transfer is also lost within the somatosensory cortex (Noctor et al. 2001; Palmer et al. 2001; McLaughlin and Juliano, 2005). Ferrets treated with MAM on E33 additionally show an increased expression of $GABA_A$ receptors ($GABA_{AR}$), which expands to upper cortical layers, and interneurons are disorganized in their laminar positions

(Jablonska et al. 2004; Poluch et al. 2008). Changes in the migratory behavior of GE-derived cells also occur (Abbah and Juliano 2012, under revision). These changes are specific to the antimitotic MAM delivery on E33, as administration of this drug on different embryonic dates leads to dramatically different results (Noctor et al. 1999; Gierdalski and Juliano 2003, 2005; Poluch and Juliano 2007, 2010). An important question emerging from these observations is whether the loss of layer 4 and subsequent redistribution of $GABA_{AR}$ and interneurons alters GABA signaling within upper cortical layers and/or on the global neocortical environment that influences the migration of late- born neurons.

Because our earlier studies in MAM-treated animals showed increased expression of $GABA_{AR}$ within the neocortex as well as an abnormal pattern of migration and distribution specifically for inhibitory cells leaving the GE, we wanted to characterize the electrophysiological properties of both the GABAergic cells migrating toward the neocortex as well as the functional impact of the changed neocortical environment on responses. To investigate if changes in cortical laminar formation alter the overall inhibitory tone within the neocortex and also affect neuronal migration, we applied whole-cell patch-clamp recording to measure spontaneous $GABA_{AR}$-mediated inhibitory postsynaptic currents (sIPSCs) in two types of cells. We studied cells migrating away from the GE relatively early during ferret cortical development (P0 – P1) and pyramidal cells of layer 2/3 of juvenile ferrets (P28 - P38). Choosing these two types of cells for functional analysis allows us to determine the effect of early environmental changes on migrating GABAergic cells and on inhibitory neurotransmission in the

somatosensory region of the resulting neocortex. Our results indicate that after E33 MAM treatment, GABAergic synaptic neurotransmission is increased significantly in cells migrating into the cortex from the GE and on cells populating the cortex in the upper layers.

Materials and Methods

Handling of animals complied with the Animal Care and Use Committee of the Uniformed Services University of the Health Sciences (USUHS). Pregnant ferrets were obtained from Marshal Farms (New Rose, NY) and maintained in the animal facilities of USUHS. On E33, ferrets were anesthetized with isofluorane (1-2%) and injected IP with 14mg/kg of MAM (Midwest Research Institute, Kansas City, MO). Ferrets were closely monitored and allowed to recover from anesthesia before returning to the animal facility to await delivery of their kits. Ferret kits were used for experiments at P0 - P1 or P28 - P38.

Preparation of Organotypic slices: Preparation of organotypic cultures followed the method described by Palmer et al. (2001). Briefly, P0 ferret kits were anesthetized with sodium pentobarbital (50mg/kg) and after observing insensitivity to pain, their brains were removed and maintained in ice-cold artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2 and 5% CO_2 under aseptic conditions in a laminar flow hood. The aCSF is composed of (in mM): 124 NaCl, 3.2 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 26 $NaHCO_3$, 1.2 NaH_2PO_4 and 10 glucose. Coronal slices of the neocortex (350-500 μm thick) were generated using a tissue chopper (Stoelting Co., Wood Dale, Illinois). Each slice was plated into a 0.4 μm culture plate insert (Millicell-CM, Bedford, MA) and placed into six-well plates containing Neurobasal media with B27, N2, and G1.2 (containing gentamycin and glutamine) supplements. Slices were incubated at 37°C with 95% O_2 and 5% CO_2.

Cell label: Migrating GE cells were labeled through electroporation using a modification of the method described by Flames et al. (2004). Transfection was achieved using a plasmid that codes for red fluorescent protein (RFP), which was cloned into pCAGGS expression vector (a gift from Dr Tarik Haydar). We applied approximately 1 µl of the plasmid DNA (5.3 µg/µl) to the ventricular region of the ganglionic eminence (GE) of organotypic slices of ferrets. The cathode of a gene paddle electrode (Harvard Apparatus, Inc., Holliston, MA) was placed within the lateral ventricle close to the GE and the anode situated close to the pia in an appropriate location; plasmid DNA was driven across the organotypic tissue wall by electric pulse generated by a BTX ECM830 pulse generator (Harvard Apparatus, Inc., Holliston, MA). A pulse of 60V current was delivered four times, each lasting for 50 ms at intervals of 950 ms (Gal et al. 2006).

Preparation of acute slices: P28-P38 ferrets were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and then decapitated. Brains were quickly removed and placed in ice-cold aCSF bubbled with 95% O_2 and 5% CO_2. The parietal cortex was blocked and serial coronal sections (400 µm thick) were cut on a vibratome (LEICA VT1000S, Buffalo Grove, IL). Slices were rapidly transferred into a holding chamber with aerated aCSF maintained at room temperature for 30 min to 1 h after dissection and before electrophysiological recordings. The aCSF was composed of (in mM): 125 NaCl, 2.5 KCl, 1.0 $CaCl_2$, 2.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 glucose.

Recordings: Whole-cell patch-clamp recordings were performed on (a) single neocortical pyramidal cells within layer 2/3 of the parietal cortex in acute

slices and (b) GE-derived cells labeled with *RFP* in organotypic slices after 14-20 days in culture. Pyramidal cells were identified by morphology; GE-derived cells were identified under fluorescent microscopy using an upright microscope with 40X objective (ZEISS Axioscope 2FS PLUS). Differential interference optics (DIC) was used for visualizing pyramidal cells. Similarly, cells migrating away from the GE were initially identified using fluorescent microscopy but subsequently visualized under DIC. Only GE cells that have crossed the corticostiatal boundary into the neocortex and display characteristic elongated leading process were selected for recording. All recordings were performed in a submersion type recording chamber where slices were continuously perfused with aerated aCSF composed of (in mM): 125 NaCl, 2.5 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 25 $NaHCO_3$, 1.25 NaH_2PO_4 and 25 glucose maintained at 28°C for pyramidal cells and 37 $^{\circ}$C for cells migrating from the GE. Slices were perfused at a rate of 3-4ml/min. Recordings were performed using a pulled borosilicate pipette filled with an internal solution composed of (in mM): 135 Cs-gluconate, 10 MgCl2, 0.1 CaCl2, 1 EGTA, 10 HEPES, 2 Na-ATP, 0.2 Na3 GTP, pH 7.3 (280–290 mOsm). In some experiments, neurobiotin at a final concentration of 0.2% was included in the internal solution for post-hoc identification. Whole-cell recordings from the soma of neuronal cells occurred in voltage-clamp mode using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) after a tight seal (1 GΩ) was formed between the recording electrode (with resistance 5-7 mΩ for pyramidal cells and 7-11 mΩ for GE-derived cells) and the cell body. For all recordings, a threshold of 2 kHz was applied to filter signals, which were

recorded and digitized (Digidata1322A, Axon Instruments), stored using pClamp software (Axon Instruments), and analyzed off-line with the Mini Analysis Program (Synaptosoft, Inc., Leonia, NJ). The access resistance was constantly monitored throughout the recording and cells that showed fluctuations beyond 15% were excluded from analysis.

Morphology: After recording, acute slices were immediately fixed in 4% paraformaldehyde overnight at 4°C. After several washes with phosphate buffered saline (PBS), slices were incubated with a solution containing 10% methanol and 0.6% hydrogen peroxide in PBS for 30 min at room temperature. After further washes with PBS, slices were incubated in a solution containing 2% bovine serum albumin (BSA) and 0.75% triton X100 in PBS for 1 h, and then in 2% BSA and 0.1 % triton X100 for 15 min. Slices were then incubated in a dilute solution of ABC elite kit overnight at 4°C. After multiple washes, cells were visualized with peroxidase staining using DAB (Immpact DAB, Vector Laboratories, Inc., Burlingame, CA.), and mounted in moviol.

Western blotting: Pieces of the neocortex cut from blocks of the parietal cortex prepared as described above were frozen on dry ice and preserved at -82°C prior to use. Protein samples were prepared first by homogenizing the tissues using RIPA lysis buffer (Santa Cruz Biotech, Santa Cruz, CA) followed by centrifugation at 14,000 g at 4°C. An estimate of the concentration of protein was obtained through colorimetric assay. Separation of proteins in the samples was accomplished by SDS-PAGE using 10% Bis-Tris gel and separated proteins were transferred to a PVDF membrane (Invitrogen, Carlsbad, CA) using

electrophoresis. A loading volume of 10 to 15 µl containing 1 µg of protein was used for each analysis. To limit non-specific binding of antibodies, membranes were initially incubated with Casein blocking buffer (PBS [0.5M NaCl] + 3% Casein + 0.5% Tween-20) for at least 2 h, followed by affinity purified rabbit polyclonal antibodies directed against $GABA_{A\alpha2}$ (1:200; ProSci Inc., Poway, CA), $GABA_{A\alpha3}$ (1:2000; Sigma, St. Louis, MO), $GABA_{A\gamma2}$ (1:400, Sigma-Aldrich, St Louis, MO.), and monoclonal anti-GAPDH (1:6000, Abcam, Cambridge, MA) for 24 h. After several washes with PBS, protein bands were detected using HRP-conjugated anti-rabbit secondary antibodies (1:1000, Jackson Lab., West Grove, PA) and HRP-conjugated anti mouse (1:6000, Thermo Scientific, Rockford, IL) and visualized using enhanced chemiluminescence detection. Signal intensities were quantified using Image j software (http://rsb.info.nih.go/ij).

Statistics: Student's t-test was applied for all statistical analysis and differences evaluated at $P< 0.05$.

Drugs: The following drugs were added to the recording buffer to isolate sIPSCs and/or mIPSCs: 50 µ M D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV), 20 µ M 5-methyl-10,11-dihydro-5H-dibenzo[a, d] cyclohepten- 5,10-imine [(+)-MK-801], both NMDA receptor antagonists, 20 µ M (_)-(2S)-5,5-dimethyl-2-morpholineacetic acid (SCH50911), $GABA_B$ antagonist; 10 µ M bicuculline methiodide (BMI), a $GABA_A$ receptor antagonist; 10 µ M 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), an AMPA/Kainate receptor antagonist, all from Tocris Cookson, Ballwin, MO, 1 µM Tetrodotoxin (TTX), a sodium channel blocker; (Sigma, St. Louis, MO).

Results

In utero exposure to MAM increases the inhibitory tone mediated by $GABA_{AR}$ in migrating cells originating from the GE.

Migrating cells leaving the GE are influenced by a variety of signaling molecules including GABA. We previously demonstrated that the normal pattern of migration and orientation of GE-derived neurons is altered in MAM-treated animals, in part due to altered $GABA_{AR}$-mediated activity (Poluch et al. 2008). To determine whether the changes in neuronal migration result from increased $GABA_{AR}$ activity in the neocortex, we evaluated $GABA_{AR}$ receptor-mediated synaptic neurotransmission in cells leaving the GE by recording the spontaneous inhibitory post synaptic currents (sIPSCs). Migrating cells were labeled with a plasmid that codes for red fluorescent protein and identified under fluorescent microscopy. Whole-cell patch-clamp recording was performed to record sIPSCs on GE cells after allowing them to cross the corticostriatal boundary into the neocortex (Figure 1E). sIPSCs were isolated using CNQX (10 μM), SCH50911 (20 μM) and AP-V (50 μM) and recorded at a holding potential (V_h) of -70mv. The amplitude and frequency of sIPSCs are increased in migrating GE cells relative to the control (Figure 1 A-D), indicating a heightened state of GABAergic neurotransmission in this population of cells.

Treatment with MAM increases the amplitude of spontaneous IPSCs in the pyramidal cells in layer 2/3 of parietal cortex.

Following treatment with MAM on E33, the thickness of layer 4 in ferret somatosensory cortex diminishes leading to reduction in the overall cortical

thickness (Noctor et al. 1999). Additionally, the distribution of $GABA_{A\alpha}$ receptor subunits is expanded and increased in the upper cortical layers; several subtypes of interneurons are also abnormally positioned (Jablonska et al. 2004; Poluch et al. 2008). To examine the functional implication of the increased expression of $GABA_{A\alpha}$ receptor subunits on the inhibitory tone within the upper cortical layers, we performed whole-cell patch-clamp recording on pyramidal cells to record sIPSCs. sIPSCs were isolated using CNQX (10 μM), SCH50911 (20 μM) and AP-V (50 μM) and recorded at a holding potential (V_h) of -70mv. We observed that in P28-38 MAM-treated animals, the amplitude of sIPSCs was significantly increased (40.08 ± 5.61 Pa, $P \leq 0.05$) relative to control animals (29.45 ± 2.31 pA, $P \leq 0.05$) (Figure 2 A, B, and C). Our data also indicate that the frequency of sIPSCs was not significantly altered following treatment with MAM (Figure 2D). The increased amplitude of sIPSCs (Figure 2C) in MAM-treated animals with no change in frequency (Figure 2D) suggests that the change in $GABA_{AR}$-mediated activity results from enhanced $GABA_{AR}$ expression in the postsynaptic membrane of layer 2/3 in the parietal cortex.

Several cells were injected with neurobiotin and reacted to visualize their morphology. An example of the types of cells from which we recorded can be seen in Figure 2E and F. All of the cells reported here were similar in morphology and position within the cortex.

In MAM-treated animals, both amplitude and frequency of miniature IPSCs were increased relative to control.

The increase in amplitude of sIPSCs indicates that $GABA_{AR}$-mediated activity is increased following treatment with MAM. The increase in sIPSC amplitude may be due to changes in postsynaptic response. To determine the underlying mechanism for increased $GABA_{AR}$-mediated activity, we recorded the action potential independent IPSCs. Miniature IPSCs (mIPSCs) were recorded in the presence of CNQX (10 μM), SCH50911 (20 μM), AP-V (50 μM) and TTX (1 μM). Our results show that the amplitude of mIPSCs increased in MAM-treated animals (17.31 ± 1.30, $n = 24, P \leq 0.05$) relative to control (13.08 ± 0.71; $n = 20, P \leq 0.05$) (Figure 3A, B, and C). Quantitative analysis of the frequency revealed a leftward shift in the inter-event interval indicating increased frequency of mIPSCs generation (Figure 3D). The addition of bicuculline eliminated this response. This result provides further indication of increased postsynaptic $GABA_{AR}$ synaptic transmission in MAM-treated animals.

The kinetics of $GABA_A$ receptors are altered in MAM-treated animals

The change in frequency of mIPSCs in MAM-treated animals suggests that alterations in presynaptic mechanisms may occur, such as alteration in number and the configuration of interneurons, whereas changes in the amplitude of response suggests changes in the postsynaptic membrane, probably due to the observed increase in expression of $GABA_A$ receptors. To further evaluate the factors responsible for these alterations, we evaluated the kinetics of $GABA_A$ receptors. First, we analyzed the 10-90 rise time and compared control with MAM-treated values. The 10-90% rise time provides spatial information about

key synaptic elements and potential alterations due to changes in presynaptic terminal number and positioning (Xie et al. 1997; Wierenga and Wadman 1999; Kobayashi and Buckmaster 2003; Alberto and Hirasawa 2010). In our study, the 10-90% rise time of the sIPSCs and mIPSCs was not significantly different between normal and MAM-treated animals (Figure 4A-I and B-I). This parameter was significantly reduced in cells migrating from the GE in our model animals during the recordings of these cells in organotypic cultures (Figure 4C-I). We next evaluated the decay time constant. In MAM-treated animals, the decay time constant of sIPSCs and mIPSCs in the pyramidal cells was significantly prolonged (Figure 4A-II and B-II). This is also true for the migrating cells originating from the GE (Figure 4C-II). Analysis of the charge transfer (measured as area) also reveals a significant increase in this parameter for the sIPSCs of the pyramidal cells in layer 2-3 (Figure 4A-III) and the cells originating in the GE (Figure 4C-III). This was also true for the recorded mIPSCs (Figure 4B-III).

Passive properties of layer 2/3 pyramidal cells and migrating GE cells are unaffected as a result of treatment with MAM.

We measured the passive properties of both pyramidal cells of layer 2/3 and migrating neurons to evaluate whether the changes in IPSC reported is related to intrinsic properties of the cell. Specifically, we recorded the capacitance, membrane potential across the cell and internal and membrane resistances. These properties provide important developmental clues relating to the level of maturity of the cells. For this analysis, we compared data obtained

from normal cells leaving the GE in organotypic cultures with cells leaving the GE after MAM treatment. We also compared pyramidal cells in normal slices with pyramidal cells in MAM-treated slices. We found no significant differences in the intrinsic properties between normal and MAM-treated cells for either the GE derived cells or pyramidal cells, suggesting that the intrinsic properties are unaffected following MAM treatment (Figure 5 A and B). In each case, there was a reduction in the membrane resistance that was notable, but not significant.

Treatment with MAM increases the expression of $GABA_{A\alpha3}$ receptor within the parietal cortex of ferrets.

The electrophysiological results presented so far support the idea that $GABA_{AR}$-mediated activity is increased following treatment with MAM. To further validate the aberrant elevation of $GABA_{AR}$ activity in our model animals, we performed western blot analysis to measure the level of expression of different subtypes of the $GABA_{AR}$ within the somatosensory cortex. We tested several receptor subtypes based on the likelihood of their expression in neocortex at this stage of development in ferrets. Although we expected that $GABA_{A\alpha1}$ receptors would be present at this age in ferret cortex, we were not able to visualize them. $GABA_{A\alpha2}$ receptors were present equally in control and MAM-treated cortex (Figure 6 top). The $GABA_{A\alpha3}$ receptor subunit, however, was increased in the parietal cortex after MAM treatment (Figure 6 middle). We also tested the presence of the $\gamma2$ subunit and found this to be slightly increased in E33 MAM-treated cortex (Figure 6 bottom). These results indicate that $GABA_{AR}$ expression

is increased, underlying the enhanced $GABA_{AR}$ -mediated synaptic transmission in MAM-treated animals.

Discussion

The current study uses a ferret model of cortical dysplasia to investigate the alterations of GABAergic synaptic transmission recorded in layer 2-3 pyramidal cells of the somatosensory cortex and on migrating GE-derived cells. We show that the basal activity mediated by $GABA_{AR}$ is enhanced within layer 2/3 and in cells leaving the GE in our model animals. An increased expression of $GABA_{AR}$ after MAM treatment is likely to underlie the changes in synaptic transmission. In our model, layer 4 formation is selectively disrupted by *in utero* administration of MAM on E33, leading to its profound reduction in size, a redistribution of interneurons, thalamic afferents, and $GABA_{AR}$ (Noctor et al. 1999; Palmer et al. 2001; Jablonska et al. 2005; Poluch et al. 2008). We also observed that cells leaving the ganglionic eminence and traveling to the neocortex after MAM treatment display abnormal dynamic properties while *en route* (Abbah and Juliano, under revision). Our earlier studies using MAM treatment during mid corticogenesis show a specific disruption of GABA-related features, leading to an apparent overall increase in GABA signaling. This study evaluated the functional implications of these findings in 2 populations of cells, GABAergic neurons leaving the GE to become interneurons and pyramidal cells in layer 2-3 of neocortex, presumably influenced by inhibitory synapses.

Treatment with MAM increases the amplitude of sIPSCs in layer 2/3 pyramidal cells.

An appropriate balance between excitation and inhibition is crucial for normal cortical function. In cortical dysplasia, this equilibrium is disrupted resulting in abnormal cortical activity and function. In pyramidal cells of layer 2/3 we observed an increased amplitude of sIPSCs without any significant change in frequency. This indicates that synaptic transmission through $GABA_{AR}$ is upregulated in MAM-treated animals, which is likely due to the increased $GABA_{A\alpha}$ receptor density on the post synaptic membrane previously observed in MAM-treated animals (Jablonska et al. 2004). To further investigate the mechanism underlying the increase in sIPSC amplitude, we evaluated the mIPSCs in the same layer 2/3 pyramidal cells. mIPSCs are caused by the quantal release of GABA from presynaptic terminals of GABAergic interneurons in the absence of action potentials (Edwards et al. 1990). The number of presynaptic terminals of interneurons and/or the postsynaptic $GABA_A$ receptor density on pyramidal cells determines the frequency of mIPSCs (Shao and Dudek, 2005). In our study, both amplitude and frequency of mIPSC were significantly increased in MAM-treated animals relative to control. The increase in mIPSCs frequency and amplitude signifies that both pre and postsynaptic mechanisms are involved in altering GABAergic neurotransmission in our model animals. Presynaptic factors such as alterations in the number of GABAergic interneurons and the quantal size of release determine the frequency of response. It is possible that the GABAergic neurons in our model are altered in some way to induce a greater number of synapses or greater quantal release. We also know that after MAM treatment, subpopulations of interneurons show altered distributions (Poluch et al. 2008).

Such redistributions may result in ectopic projections or altered synapses with upper cortical layer pyramidal cells. Such region-specific alterations in local circuit connections occur in other models of cortical dysplasia (Brill and Huguenard 2010). Postsynaptic mechanisms such as the density and internalization of postsynaptic protein modulate the size of GABAergic neurotransmission contributing to the change in mIPSC amplitude (DeFazio and Hablitz, 1999; Kittler et al. 2000; Loup et al. 2006; Goodkin et al. 2007; Michels and Moss, 2007; Hartmann et al. 2008). The frequency of sIPSCs, which is the summation of both action potential dependent and independent events, was not altered even though the frequency of mIPSC was increased in MAM-treated animals. A number of factors may be responsible for this, notably the nature of the network of interneurons within layer 2/3 (Tamas et al. 1998; Bacci et al. 2003).

Although reduction in amplitude and frequency of IPSCs are frequently associated with epilepsy and cortical dysplasia (McDonald et al. 1991; Henry et al. 1993; Buckmaster and Dudek 1997; Hablitz and Defazio, 1998; Jacobs et al. 1999; Rocha et al. 2007), the converse occurs in other models of these diseases. Our finding of increased GABAergic neurotransmission in layer 2/3 pyramidal cells corresponds with a recent finding of enhanced GABAergic activity in pediatric cortical dysplasia (Cepeda et al. 2012). Other studies demonstrate a similar increase in GABAergic activity. A freeze lesion model of cortical dysplasia reports increased IPSCs (Prince et al. 1997; DeFazio and Hablitz 1999; Brill and Huguenard 2010). In other models of cortical dysplasia, however, a reduction in

inhibitory tone occurs, such as *in utero* exposure to carmustine, MAM, or radiation in rats (Xiang et al. 2006; Zhou et al. 2009; Zhou and Roper 2010; Karlsson et al. 2011). In these models, the development of seizures is believed to be a consequence of reduced inhibitory drive similar to epilepitogenic foci reported in animal models of epilepsy (Rice et al. 1996; Hirsch et al. 1999; Cossart et al. 2001; Kobayashi and Buckmaster 2003; Shao and Dudek 2005; Sun et al. 2007). This analysis is complicated further by evidence of elevated GABAergic signaling in other seizure models (Gibbs et al. 1997; Nusser et al. 1998; Cossart et al. 2001; Shao and Dudek 2005; Zhan and Nadler 2009).

We did not observe any significant variation in the passive properties between MAM-treated and sham animals indicating that the cellular intrinsic properties remain relatively unaffected by MAM treatment. For both the cells migrating away from the GE and the layer 2-3 pyramidal cells, however, the membrane resistance was lower after MAM treatment, compared with control, although the difference was not significant. It is not clear what might cause this change, but it is possible that the membrane characteristics of MAM-treated pyramidal cells have properties that would cause a slight difference in membrane resistance. One possibility is that the MAM-treated brains may mature at a different rate than the normal brains, leading to small distinctions in membrane characteristics (Goldberg et al. 2011).

$GABA_A$ receptor-mediated activity is enhanced in migrating GE-derived neurons after MAM treatment.

The orientation and distribution of interneurons is altered in E33 MAM-treated animals, in part due to elevated $GABA_{AR}$-mediated activity (Poluch et al., 2008). Recently, we found that elevated $GABA_{AR}$-mediated activity plays a role in altering the kinetic behavior of migrating cells leaving the GE (Abbah and Juliano, under revision). To determine whether the cells leaving the GE receive abnormally increased $GABA_{AR}$ signaling after MAM treatment, we measured the sIPSCs. Both amplitude and frequency of sIPSCs were increased in MAM-treated animals suggesting that $GABA_{AR}$ synaptic transmission enhances in MAM-treated animals. GABA signaling plays an important role in promoting migration of GE cells and alterations in the basal level of $GABA_{AR}$ activity is likely to be deleterious in the adequate migration of GE-derived cells. Specifically, elevated $GABA_{AR}$-mediated activity, while initially promoting migration of cells derived from the GE, triggers early maturation of these cells by inducing precocious expression of the potassium chloride co-transporter (KCC2) (Ganguly et al., 1999; Abbah and Juliano, in revision). During development, KCC2 maintains a high extracellular Cl^- gradient and induces hyperpolarization of cells in response to GABA, playing a role in the developmental switch from the GABA-induced depolarization to hyperpolarization, which may also terminate cell migration (Rivera et al, 1999; Bortone and Polleux, 2009). Thus the elevated functional responses to $GABA_{AR}$ synaptic transmission in cells migrating away from the GE supports the idea that an aberrant GABAergic environment contributes to both abnormal migration, and disrupted processing of information.

Kinetics of $GABA_A$ signaling is altered in MAM-treated animals.

The measure of 10-90% rise time gives relative spatial information with respect to postsynaptic sites; changes in rise time deduce the locus of change in presynaptic terminal number. It is widely accepted that receptors located in the soma of postsynaptic membrane give a faster rise time compared to those located on the dendrites (Shao and Dudek 2005). In our study there is no significant change in the rise time in either the sIPSCs or the mIPSCs of pyramidal cells in layer 2/3 indicating that there is no significant positional change in the active zones and receptor clusters between normal and MAM-treated animals (Xi et al. 1997; Alberto and Hirasawa 2011). In the cells migrating from the GE, however, we observed a significant reduction in rise time after MAM treatment compared to normal. Although, the reason for this change is unclear, one factor that may be responsible is the relative position of the migrating cells in the developing neocortex, which may affect the nature of synaptic contact they receive. The decay time constant is influenced by the density of postsynaptic receptors and receptor subtype composition (Xi et al. 1997; Shao and Dudek 2005); prolongation of decay time constant that we observed in both the sIPSCs and mIPSCs in MAM-treated animals is consistent with increased expression of $GABA_{AR}$. The amplitude of the IPSCs and decay time constant determines the charge transfer. In our study, the charge transfer was significantly increased for both sIPSC and mIPSC in cells leaving the GE and layer 2/3 pyramidal cells following treatment with MAM. This is consistent with the increased amplitude and decay time that we reported.

Expression of $GABA_A$ receptor subunits are altered in MAM-treated animals.

Our results show that GABAergic neurotransmission is enhanced in the neocortex of MAM-treated animals, probably as a result of increased expression of $GABA_{AR}$. We also demonstrated that $GABA_{AR}$ are increased after E33 MAM treatment, primarily in the upper cortical layers using receptor binding and immunohistochemistry (Jablonska et al. 2004). Here we also find an increase in the amplitude of sIPSCs and mIPSCs in pyramidal cells of layer 2/3 and a prolonged decay time. To further confirm that increased $GABA_{AR}$ receptor expression is responsible for the enhanced GABAergic neurotransmission, we applied western blot techniques to measure the level of various subunits of $GABA_A$ receptors. Our data shows that $GABA_{A\alpha3}$ expression is increased in dysplastic cortex compared to control. The mammalian $GABA_{AR}$ is made of five subunits generated from 19 possible subunits (α1-6, β1-3, γ1-3, δ, ε, θ, ρ, and ρ1-3) - (Olsen and Sieghart 2008). Compelling evidence indicates that these subunits have differences in temporal expression and regional distribution during development (Garrett et al. 1990; McKernan et al. 1991; Araki et al. 1992; Laurie et al. 1992; Fritschy et al. 1994; Pirker et al. 2000; Yu et al. 2006; Hashimoto et al. 2009; Carlson et al. 2010). $GABA_{A\alpha3}$ subunit is expressed early in development and mediates a number of the developmental activities of GABA (Laurie et al. 1992; Poulter et al. 1992; Fritschy et al. 1994; Carlson et al. 2010). The increased expression of $GABA_{A\alpha3}$ appears to underlie the enhanced

GABAergic neurotransmission reported here. We also observed increased expression of $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ subunits in the neocortex of MAM-treated animals at P0 (Abbah and Juliano, under review). A possible explanation for increased $GABA_{AR}$ expression in layer 2/3 of parietal cortex in MAM-treated animals is the redistribution of thalamic afferents, which associate closely with $GABA_{AR}$ during development (Noctor et al. 2001; Palmer et al. 2001; Jablonksa et al. 2004). In normal animals, thalamic afferents make synaptic contacts primarily in layer 4 (Jones and Burton 1976). This ensures that afferent information to the neocortex is first received in layer 4 before being transferred to the upper layers and lower layers 5 and 6 (White 1989; Senft and Woolsey 1991; Johnson and Alloway 1996). Thus, in the presence of diminished layer 4, the thalamic afferents and associated $GABA_{AR}$ become redistributed to upper cortical layers; this thalamic redistribution may account for the increase in GABAergic signaling in this region. Others report a close correlation between the expression of $GABA_{AR}$ and projection of thalamic afferents in the somatosensory and visual cortices of rodents (Schlaggar et al. 1994, Broide et al. 1996). Thalamic afferents also play a significant role in the expression and regional distribution of $GABA_{AR}$ subunits within the visual and somatosensory cortical areas of rodents (Paysan et al. 1997). In rats, the expression of the α1 subunit, the predominant subunit of $GABA_{AR}$ in adult animals, is highest within the primary somatosensory (S1) and visual (V1) cortical areas; these regions also receive substantial terminations of thalamic afferents (Fritschy et al. 1994; Paysan et al. 1994). In S1, the distribution of α1 receptors mirrors the arrangement of the barrels, the cortical

representation of whiskers. On the other hand, α2, α3 and α5 subunits have low expression in S1 but high expression in the association areas of the somatosensory cortex. Interestingly, perturbation of the thalamic afferents early during development differentially impacts the density and regional distribution of various α subunits within the parietal cortex (Paysan et al. 1997).

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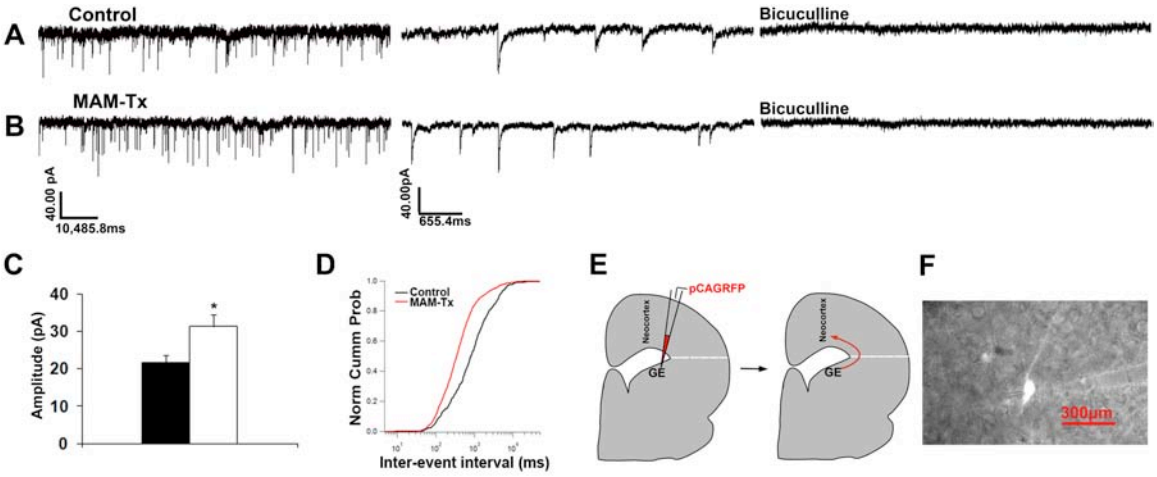

Figure 1. In MAM-treated animals, both amplitude and frequency of sIPSC is increased in migrating GE cells within the developing cortex. sIPSCs were recorded in a voltage clamp mode at a holding potential of -70mV and isolated using CNQX (10 μm), SCH50911 (20 μm), AP-V (50 μm) and TTX (1μm). Representative traces of sIPSCs recorded in a cell leaving the GE in normal (A) or MAM-treated cortex. (B). sIPSCs in both normal and MAM-treated GE cells were blocked by bath application of 10 μm bicuculline. (C) The amplitude of the sIPSCs is significantly increased in MAM-treated animals relative to control. (D) The cumulative probability plots of sIPSC frequency show a left-ward shift following treatment with MAM, indicating a reduction in inter-event interval and increase in frequency. (E) A schematic diagram of an organotypic slice illustrating the positioning of the *RFP* construct in a pipette and the migratory path of the cells exiting the GE. (F) Image of a cell migrating away from the GE during whole-cell patch-clamp recording. An arrow points to the recording electrode. For group analysis of amplitude and frequency of sIPSC, *n*= 16 cells (normal) and 21 cells (MAM-treated), *$P< 0.05$, Student T-test. Error bars = Standard error of mean.

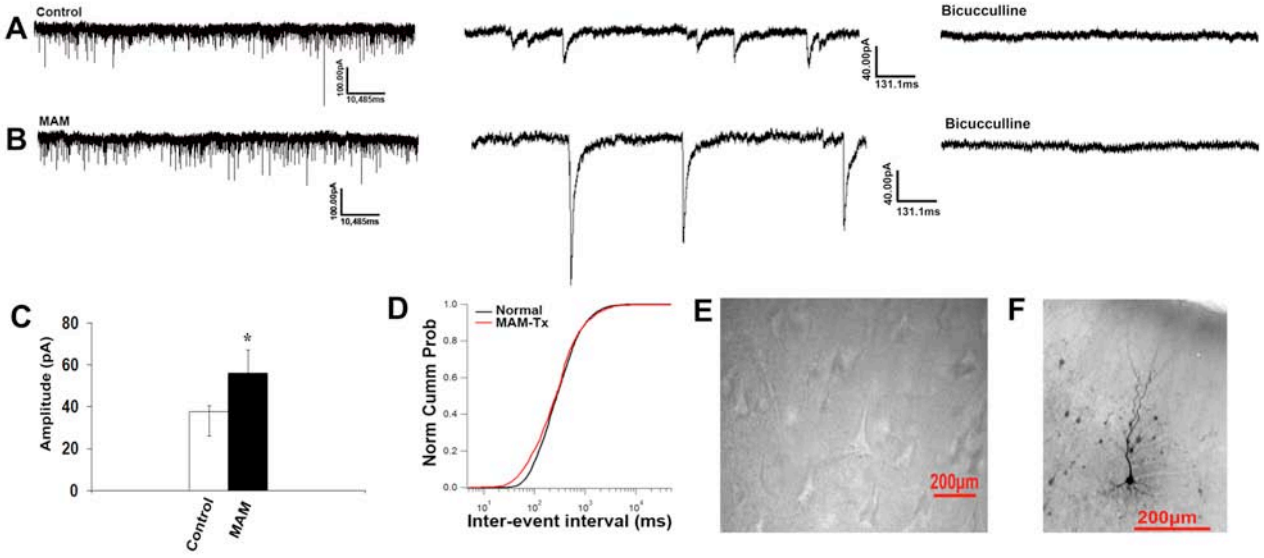

Figure 2: Amplitude but not frequency of spontaneous IPSCs (sIPSCs) are increased in pyramidal cells of layer 2/3 of ferret parietal cortex. sIPSC were recorded in voltage clamp mode at a holding potential of -70mV in the presence of CNQX (10 µm), SCH50911 (20 µm), AP-V (50 µm). (A) Representative traces of recorded sIPSCs in pyramidal cells of layer 2/3 of normal cortex. (B) Representative traces of recorded sIPSC in pyramidal cells of layer 2/3 of a MAM-treated cortex. sIPSCs in both normal and MAM-treated pyramidal cells were blocked by bath application of 10 µm of bicuculline. (C) The amplitude of sIPSC is significantly increased in MAM-treated animals relative to control. (D) The cumulative probability curve of the frequency of sIPSCs indicates that the inter-event interval was not altered following treatment with MAM. For group analysis of amplitude and frequency of sIPSC, *n*= 40 cells (normal) and 24 cells (MAM-treated), * *P< 0.05*, Student T-test. Error bars = Standard error of mean. (E) Example of a pyramidal cell in parietal cortex during whole-cell patch-clamp recording. (F) Post-hoc identification of pyramidal cell in layer 2/3 of the parietal cortex after whole-cell patch-clamp recording.

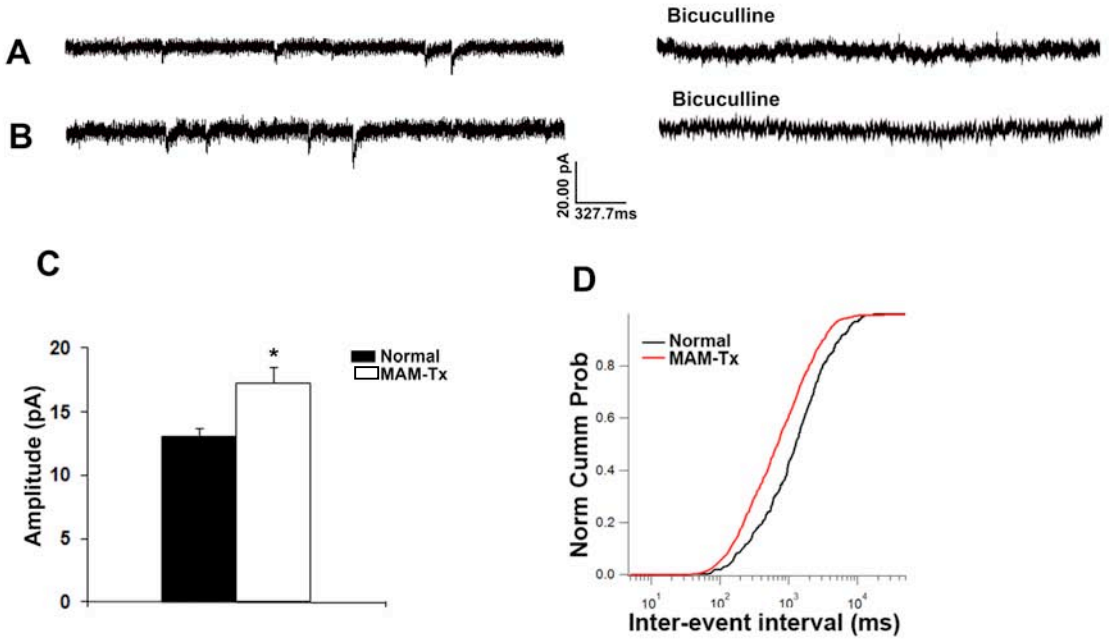

Figure 3. In MAM-treated animals, both amplitude and frequency of miniature IPSC (mIPSC) is increased in pyramidal cells of layer 2/3 of ferret parietal cortex. mIPSCs were recorded in voltage clamp mode at a holding potential of -70mV and isolated using CNQX (10 μm), SCH50911 (20 μm), AP-V (50 μm) and TTX (1μm). (A) Representative traces of mIPSCs recorded in pyramidal cells of layer 2/3 of a normal cortex. (B) Representative traces of mIPSCs recorded in pyramidal cells of layer 2/3 of a MAM-treated cortex. mIPSCs in both normal and MAM-treated pyramidal cells were block by application of 10 μm bicuculline. (C) The amplitude of the mIPSCs is significantly increased in MAM-treated animals relative to control. (D) Cumulative probability plots of frequency of mIPSC show a left-ward shift following treatment with MAM indicating a reduction in inter-event interval and increase in frequency. For group analysis of the amplitude and frequency of mIPSCs, *n*= 20 cells (normal) and 24 cells (MAM-treated), * $P < 0.05$, Student T-test. Error bars = Standard error of mean.

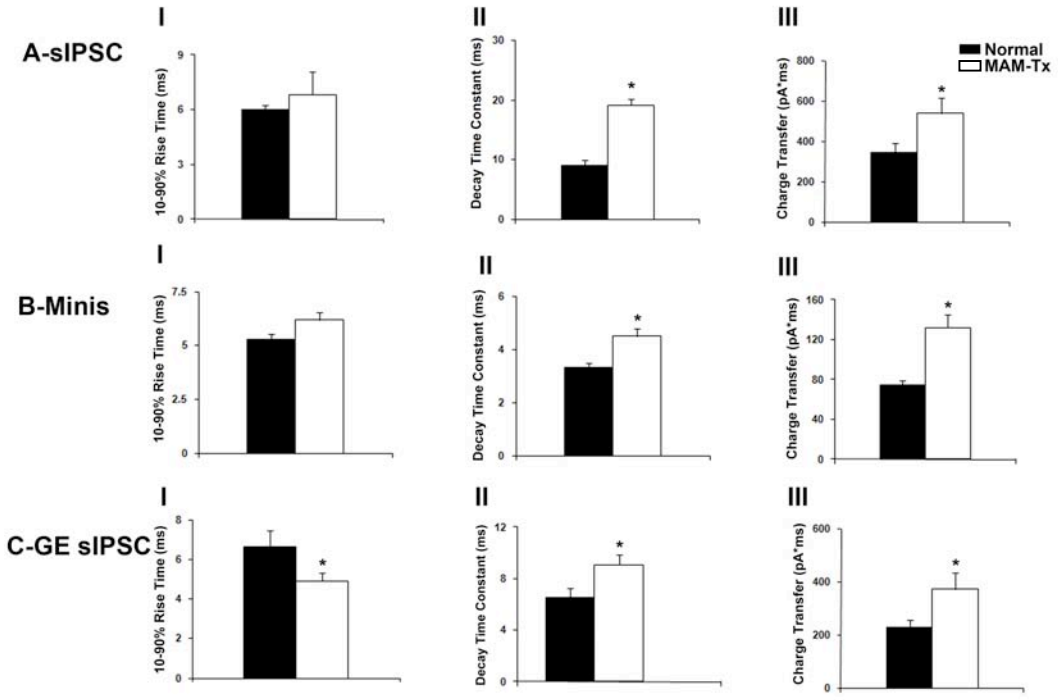

Figure 4. Kinetics of $GABA_A$ receptors are altered following treatment with MAM. (A) In pyramidal cells, the 10-90 rise time of the sIPSCs was not significantly altered following treatment with MAM (I). However, the decay time constant was significantly prolonged (II); the charge transfer was also significantly increased in MAM-treated animals (III). *n*= 40 cells (normal) and 24 cells (MAM-treated), **P*< *0.05*, Student T-test. (B) Analysis of $GABA_R$ kinetics of the mIPSCs indicates that the 10-90% rise time was unchanged in MAM-treated animals (I). The decay time constant, however, was significantly prolonged (II) and the charge transfer also significantly decreased (III). *n*= 20 cells (normal) and 24 cells (MAM-treated), **P*< *0.05*, Student T-test. (C) Kinetics of $GABA_R$ in migrating GE cells. (I) The 10-90% rise time of recorded sIPSC in migrating GE cells was shortened in MAM-treated animals relative to control. The decay time constant (II) and charge transfer (III) however, increased significantly. *n*= 16 cells (normal) and 21 cells (MAM-treated), **P*< *0.05*, Student T-test. Error bars = Standard error of mean

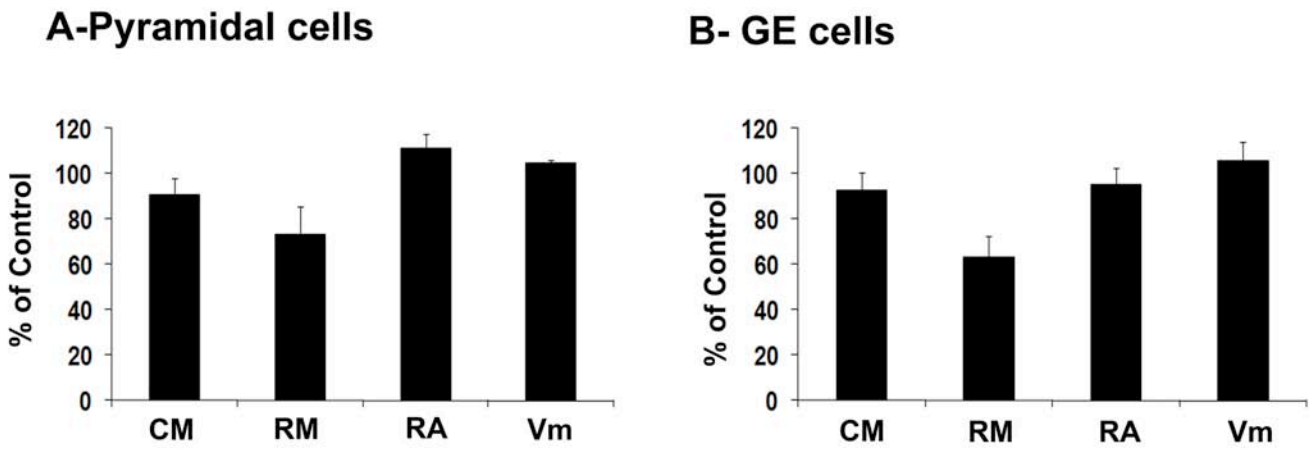

Figure 5. Treatment with MAM did not significantly alter the passive properties of both pyramidal cells of layer 2/3 and migrating GE-derived cells. (A) Group data from MAM-treated pyramidal cells (n= 14) normalized relative to control animals. (B) Group data from MAM-treated migrating GE cells (n= 13) normalized relative to control animals. $P< 0.05$, Student T-test. Error bars = Standard error of mean.

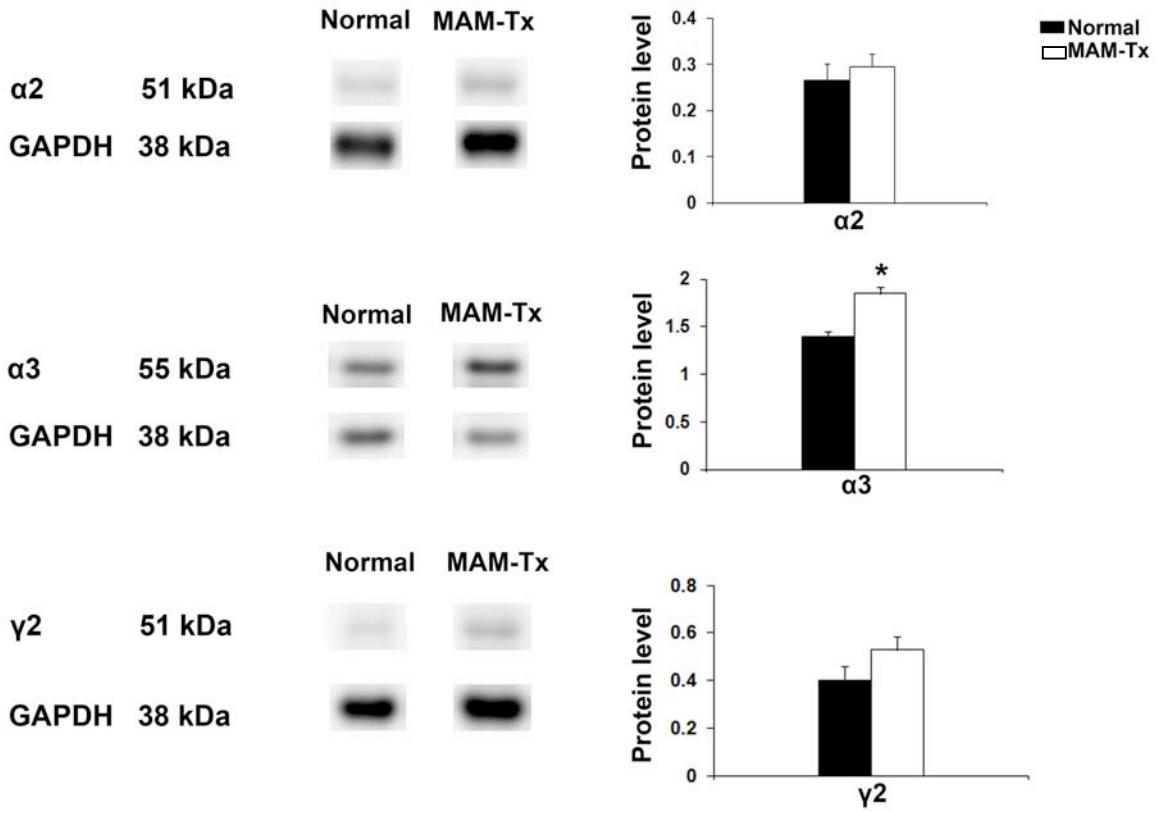

Figure 6. Expression of $GABA_A$ receptor subunits following treatment with MAM. Treatment with MAM results in a significant increase in the expression of $GABA_{A\alpha3}$ receptor subunits and slight elevation of $GABA_{A,2}$ receptor subunits, but those of $GABA_{A\alpha2}$ subunits were unchanged, in the neocortex as demonstrated by Western blots. Protein bands were detected through enhanced chemlumniscence and quantitative analysis of the bands performed by densitometric analysis using image J software for both normal

CHAPTER 4

DISCUSSION

Cortical dysplasia (CD) is a developmental abnormality characterized by changes in structure and function of the cerebral cortex. CD often results from the failure of immature neurons to migrate appropriately to their cortical targets. This failure compromises the establishment of the six-layered structure of the cortex and impairs its ability to process information and perform its role to integrate and command. The lack of a satisfactory procedure for managing disorders due to CD underlies the need for development of alternative approaches; this requires critical insight into the mechanisms underlying neuronal migration to comprehend the many disorders that result from disrupted migration. Although recent studies improve our understanding of neuronal migration, significant gaps in knowledge still remain. Moreover, most of these studies were performed in lower animals and rodents, which have important differences compared with higher gyrencephalic mammals like humans and thus necessitate the study of neuronal migration in more advanced animals.

We developed a model of cortical dysplasia in ferrets by interrupting the development of layer 4 cells, which results in abnormal placement of specific populations of interneurons and the redistribution of thalamic afferents and $GABA_A$ receptors (Noctor et al., 1999; Jablonska et al, 2005; Poluch et al., 2008). We demonstrate that the dynamic movement of migrating GE-derived cells is impaired in a ferret model of cortical dysplasia in part due to enhanced $GABA_A$

receptor ($GABA_{AR}$) transmission. We also demonstrated that changes in cortical lamination in our model animals impact the microcircuit in upper cortical layers to alter the balance between excitation and inhibition.

Treatment with MAM alters the kinetic behavior of GE-derived cells

In our first set of experiments, we investigated the impact of MAM-treatment on the dynamic pattern of movement of both GE cells and CVZ cells. During development, a well regulated process of neuronal migration involving both intrinsic factors and environmental cues ensures that projection neurons and interneurons that make up each layer of the cortex reach their appropriate targets to create the normal laminar pattern of the cortex (Anderson et al, 1997; Powell et al, 2001; Lopez-Bendito et al., 2003 and 2008; Polleux et al, 2003; Stumm et al., 2003; Alifragis et al., 2004; Flames et al., 2004; Cuzon et al, 2006; Cobos et al., 2007; Liodis et al., 2007; Friocourt et al., 2008). Changes in the proper function of any of the numerous factors that guide migration can lead to abnormal cell positioning and CD. In our model of CD, the neocortical environmental is altered as a result of redistribution of thalamic afferents, $GABA_A$ receptors, and a sub-population of interneurons, following the loss of layer 4 (Noctor et al., 1999; Jablonska et al., 2005; Poluch et al., 2008). To better understand the mechanisms contributing to the redistribution of interneurons, we evaluated several parameters of kinetic behavior in migrating GE cells including speed and exploratory tendencies. Cells leaving the GE in normal animals migrated significantly faster compared to similar cells in MAM-treated animals. In addition,

GE generated neurons in MAM-treated animals exhibited less exploration of the neocortical environment. Exploratory behavior, including extension of multiple leading processes and changes in direction of movement, results from coordinated signaling from various sites, which communicate cues that determine the final location of these cells. The change in the speed of migration and exploratory activity of interneurons following treatment with MAM therefore has important implications on the overall positioning of cells within the neocortex.

Is the impact of MAM treatment restricted to cells generated in the GE?

To determine if the effect of E33 MAM treatment on the kinetics of migration is restricted to interneurons alone, we also investigated the dynamic pattern of migration of neurons arising from the cortical VZ. Although the migration speed of CVZ cells was reduced in MAM-treated animals, the deficit was not significant. We also did not observe any significant differences in exploratory behavior between cells arising from the CVZ in normal animals and those receiving E33 MAM treatment, indicating that the impact might be restricted to tangentially migrating GABAergic interneurons. The differential impact of MAM-treatment on the kinetic behavior of GE and CVZ-derived cells relative to normal cells is not surprising since these two cell populations have different neurogenic origin and migrate under the influence of similar but not identical signaling mechanisms (Marin and Rubenstein, 2003). For instance, CVZ cells, which are derived from RG cells, rely predominantly on the structural support of RG for their radial migration (Noctor et al., 2001, 2004). Migration of GE-derived cells is largely

independent of radial glia support. In addition, different transcription programs regulate migration of CVZ and GE-derived cells. Furthermore, different signaling mechanisms provide positional guidance to these two population of cells within the neocortex (for details, see above).

Does treatment with MAM alter the functional properties of migrating GE cells?

Next, we investigated the impact of MAM treatment on the $GABA_A$ receptor activity of migrating GE cells. The orientation and distribution of interneurons is altered in MAM-treated animals in part due to elevated $GABA_A$-mediated activity (Poluch et al, 2008). To determine whether migrating GE cells receive abnormally increased $GABA_{AR}$ signaling, we recorded sIPSCs using whole-cell patch-clamp recording. Both the amplitude and frequency of sIPSCs were increased in MAM-treated animals suggesting that $GABA_{AR}$-mediated synaptic transmission is enhanced in our model animals. sIPSC amplitude is influenced by presynaptic factors such as number of inhibitory interneurons in synaptic contact with migrating GE cells as well as the density of $GABA_{AR}$ expressed on the membrane of GE cells migrating to the neocortex (Braga et al., 2003; Shao and Dudek 2005). The frequency is determined by number of presynaptic GABAergic neurons. The GE cells that we recorded from were still in transit enroute to their target destination in the neocortex. Consequently, synaptic contacts with these cells are transient and different regions of the neocortical environment or route of transit influence the level of GABA signaling. In our model, loss of layer 4 leads to redistribution of thalamic afferents and associated $GABA_{AR}$; this alters the

neocortical environment precisely with respect to GABA signaling to influence migration and positioning of migrating GE cells. The increase in both amplitude and frequency of sIPSCs suggests that both pre-synaptic mechanisms and postsynaptic $GABA_{AR}$ expression contribute to the elevated $GABA_{AR}$-mediated increase in inhibitory synaptic neurotransmission.

Abnormal elevation in $GABA_{AR}$-mediated activity may underlie the altered migration pattern in GE-derived cells.

The increase in amplitude and frequency of sIPSCs in cells migrating away from the GE suggests that inhibitory neurotransmission received by GE-derived cells is elevated in E33 MAM-treated animals. In the second set of experiments, we examined the relationship between the abnormal increase in $GABA_{AR}$-mediated activity and the alteration in GE cell migration. First, we evaluated the basal level of $GABA_{AR}$ by measuring the expression of $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ subunits in the neocortex. The mammalian $GABA_{AR}$ is made of five subunits generated from 19 possible subunits (α1-6, β1 -3, γ1 -3, δ, ε , θ , ρ , and ρ1 -3) - a pentameric hetero-oligomer (Olsen and Sieghart, 2008). There is compelling evidence based on work from various labs indicating that these subunits have different temporal expression and regional distribution during development (Garrett et al., 1990; Fritschy et al., 1994, Yu et al., 2006; Araki et al., 1992; Laurie et al., 1992; Hashimoto et al., 2009; McKernan et al., 1991; Pirker et al., 2000). Both α2 and α3 subunits of the $GABA_A$ receptor are expressed early during development and may mediate the initial roles of GABA during development that includes

regulation of DNA synthesis, cell proliferation, migration, differentiation, development of AMPA receptors, excitatory synapse formation and possibly acting as a stop signal for migrating GE cells (Poulter et al., 1992; Barbin et al., 1993; Heck et al., 2007; LoTurco et al., 1995; Wang and Kriegstein, 2008, 2009; Bortone and Polleux, 2010; Inada et al., 2011). In our study, $GABA_{A\alpha2}$ and $GABA_{A\alpha3}$ expression are increased in the neocortex of young (P0 – P1) MAM-treated animals compared to controls. We did not observe any noticeable expression of the α1 subunit in the neocortex of both normal and MAM-treated animals at this age. This is not surprising since this subunit typically show delayed expression during development (Araki et al., 1992; Laurie et al., 1992). The expression of the α1 subunit is accompanied by decrease in the expression of the α2 and α3 subunits; this transition occurs as animals mature (Fristchy et al., 1992).

GABA initially acts as an excitatory neurotransmitter during development to facilitate cell migration, but switches to mediate inhibition as cells mature (Plotkin et al., 1997; Clayton et al., 1998; Rivera et al., 1999). The dual role of GABA is due to the sequential expression of NKCC1 and KCC2. Migrating neurons initially express NKCCl, which imports chloride ions (Cl^-) into the cell to maintain a higher intracellular Cl^- concentration (Clayton et al., 1998). In response to $GABA_{AR}$ activation, the outward movement of Cl^- results in depolarization of the cells and triggers Ca^{2+}-mediated events that influence the cytoskeleton and stimulate migration (Behar et al., 2001; Soria & Valdeolmillos, 2002). As cells mature, NKCCl downregulates and coincides with increased

expression of KCC2 (Plotkin et al., 1997). KCC2 is a Cl^- exporter that maintains a higher extracellular gradient of Cl^-. Consequently, $GABA_{AR}$ activation when KCC2 is increased results in hyperpolarization and cell inhibition (Payne, 1997; Rivera et al., 1999). Interestingly, in dissociated hippocampal cell cultures, activation of $GABA_{AR}$ induces the expression of NKCCl and KCC2 (Ganguly et al., 2001). In addition, pharmacologic blockade of $GABA_A$ delayed the expression of KCC2, while increased activity of $GABA_{AR}$ induces precocious expression of the transporter. Premature expression of KCC2 as a result of a heightened state of $GABA_{AR}$-mediated activity, as we see here, may be responsible for the migration defects observed in MAM-treated ferrets. KCC2 is implicated in several important processes, including decreased motility as a neuron migrates, which leads to cell slowing, stopping, and differentiating. As a consequence, a premature switch of the $GABA_A$ response may occur and produce early arrest of migrating interneurons. Bortone and Polleux (2009) demonstrated that depolarization through $GABA_A$ receptors stimulates motility of GABAergic migrating neurons while hyperpolarization via $GABA_{AR}$, induced by KCC2 expression, provides a stop signal for migrating cells. We observed an increased expression of both KCC2 monomer and oligomers in the neocortex of young MAM-treated animals. The ratio of KCC2 monomer to oligomer was also reduced in MAM-treated animals, which indicates greater activity of this transporter in our model animals. Oligomerization of KCC2 is an important step in the activation and function of KCC2 as a Cl^- exporter (Blaesse et al., 2006). These overall changes in KCC2 expression, as a consequence of increased $GABA_{AR}$ actions, may result in a

slowing of interneuron migration, causing early differentiation and maturation of GE-derived cells.

Does blocking $GABA_{AR}$ activity ameliorate the deficit in interneuron migration in MAM-treated animals?

GABA is an important influence on migrating cells, although its precise role in the migration of telencephalic cells is complex. GABA receptors are important during different phases of migration and mediate aspects of the initiation, continuation, and termination of cellular movement (Behar et al., 1996, 2001; Bolteus and Bordey, 2004; Heck et al., 2007; Bortone and Polleux, 2009). Cuzon and colleagues (2006) report that ambient $GABA_A$ activity is present throughout the path of migration of mouse GE cells into the neocortex, but increases in activity as neurons approach and enter their cortical target. Our studies suggest that MAM treatment results in increased ambient $GABA_{AR}$-mediated activity, which may interfere with migration of GE cells as they move *en route* to the neocortex. The region between the cortical striatal junction and developing cortical plate is identified by Cuzon et al (2006) as a site of normally high levels of GABA, which act as an important factor in the proper movement of GE derived cells. This is supported by our original observation of expanded $GABA_{A,\alpha}$ immunoreactivity in MAM-treated cortex (Jablonska et al., 2004) and the current finding of increased $GABA_{A,\alpha 3}$ receptor subunit as demonstrated by Western blot. Both α2 and α3 subunits of the $GABA_A$ receptor are expressed early during development and may mediate the initial roles of GABA during development (Laurie et al., 1992;

Poulter et al., 1992; Fritschy et al., 1994; Carlson et al., 2010). This assists in explaining our finding that elevated levels of GABA interfere with migration of GE cells to slow the pace but not prevent their movement.

To investigate the involvement of $GABA_{AR}$-mediated transmission in the migration of interneurons, we manipulated the level of $GABA_{AR}$ activity in MAM-treated organotypic cultures using BMI to reduce the ambient $GABA_{AR}$ activity. We predicted that limiting the abnormal increased $GABA_{AR}$ activity would change the cellular actions in MAM-treated cortex to be more like normal. In fact, blocking $GABA_{AR}$ activity in MAM-treated animals resulted in significant enhancement of the migration speed of cells leaving the GE as well as the percentage of MAM-treated GE cells extending more than one leading process. This suggests that reducing $GABA_{AR}$-mediated sgnalling in the MAM-treated ferret encourages migratory activity to become more normal.

What is the functional implication of impaired migration of GE-derived neurons?

The data we present here indicates that elevated $GABA_{AR}$-mediated activity in MAM-treated animals plays a role in the impaired migration of GE-derived cells. An important question that arises from this finding is what impact these changes have on GABAergic neurotransmission in the resulting cortex, which then influences function. We addressed this question by applying whole-cell patch clamp recording to measure IPSCs in pyramidal cells in layer 2/3. We observed increased amplitude of sIPSCs in pyramidal cells of layer 2/3 without any

significant change in frequency. This indicates that $GABA_{AR}$-mediated activity is higher in MAM-treated animals. To investigate the mechanism underlying the increase in sIPSC amplitude, we evaluated the mIPSC in layer 2/3 pyramidal cells. mIPSCs are caused by quantal release of GABA from presynaptiic GABAergic interneurons in the absence of action potentials and thus reflect release probability of GABA (Edwards et al., 1990). The number of presynaptic terminals of interneurons and/or postsynaptic $GABA_{AR}$ density on pyramidal cells determines the amplitude and frequency of mIPSC (Shao and Dudek, 2005). In our study, both amplitude and frequency of mIPSC were significantly increased in MAM-treated animals relative to control. The increase in mIPSC frequency and amplitude that we observed here signifies that both pre and postsynaptic mechanisms may be involved.

We also observed changes in the kinetics of opening of $GABA_{AR}$ in our model animals. The 10-90% rise time gives relative spatial information with respect to postsynaptic sites and changes in rise time could be used to deduce the locus of change in presynaptic terminal number. It is widely accepted that receptors located in the soma of postsynaptic membrane give a faster rise time compared to those located on the dendrites (Shao and Dudek, 2005). In our study there is no significant change in the rise time in the sIPSC and mIPSC of pyramidal cells of layer 2/3 indicating no significant positional change in the active zones and receptor clusters between normal and MAM-treated animals (Xie et al., 1997; Alberto and Hirasawa, 2011). In the migrating GE cells, however, we observed a significant reduction in rise time. Although, the reason

for this change is unclear, one factor that may be responsible is the relative position of the migrating GE cells in the emerging neocortex that may affect the nature of synaptic contact they receive. The decay time constant is influenced by the density of postsynaptic receptors (Xi et al., 1997, Shao and Dudek, 2005); prolongation of decay time constant that we observed in both sIPSC and mIPSC in MAM-treated animals is consistent with increased expression of $GABA_A$ receptors. The amplitude of IPSC and decay time constant determines the charge transfer. In our study, the charge transfer was significantly increased in the sIPSC and mIPSC recorded in both migrating GE cells and layer 2/3 pyramidal cells following treatment with MAM suggests. This is consistent with the increased amplitude and decay time that we reported.

Expression of $GABA_A$ receptor subunits is altered in dysplastic cortex

The result we present above suggests that GABAergic neurotransmission is enhanced in the neocortex of MAM-treated animals, probably as a result of the increased expression of $GABA_A$ receptors. This is evidenced by the increased amplitude of sIPSCs and mIPSCs in pyramidal cells of layer 2/3 and the prolonged decay time in animals P28-38. To further confirm that increased $GABA_{AR}$ expression is responsible for the enhanced GABAergic neurotransmission, we applied western blot to measure the level of various subunits of $GABA_{AR}$ in the older cortex. Our data shows that $GABA_{A\alpha 3}$ expression was significantly increased in our model animals, as it was in the P0 animals. We also observed a slight increase in the expression of $GABA_{A\gamma 2}$ and no change in

the $GABA_{A\alpha 2}$ subunits in dysplastic cortex compared to control. The increase in $GABA_{A\alpha 3}$ receptor subunit may underscore the elevated GABA transmission in pyramidal cells of MAM-treated juvenile ferrets. The $GABA_{A\gamma 2}$ receptor subunit is presumed to also be important for synaptic transmission and for the postsynaptic clustering of $GABA_{AR}$ during the development of synapses (Schweitzer et al., 2003). In this regard, it could clearly play a role in the abnormal synaptic transmission observed here. It is not clear why we did not observe an increase or change in the $\alpha 1$ receptors in either the P0 – P1 or P28 – P38 animals, as we would expect these to increase as the animal matures. It may be that the ferret neocortex is still relatively immature, even at P28 – P38, and does not express high levels of this receptor until a later date. In any case, the obvious increase and probable change in the composition of at least 2 $GABA_{AR}$ subtypes can clearly underlie the change in kinetics that we observed in our recordings.

Conclusion and future directions.

In this set of experiments, we demonstrated that *in utero* admistration of MAM on E33 leading to development of CD, selectively interfers with migration of GE-derived cells by altering their kinetic properties. We also showed that the aberrant migration of MAM-treated GE cells results from anomalous increase in $GABA_{AR}$ synaptic transmission within the neocortical environment. This increase appears to enhance the rate of maturation of GE derived cells by inducing the precocious expression of KCC2 (Figure 4). As a result, GE-derived neurons migrate slowly and terminate at aberrant sites within the neocortex; resulting in a

change in cortical microcircuitry that impairs information processing within the cortex. This finding provides evidence that abnormal elevation in GABA signaling during neocortical maturation may underlie the development of CD.

Several neuropsychiatric disorders such as epilepsy, schizophrenia, mental retardation and autism result from abnormal cortical development. Most of these disorders present with impaired GABA function due to loss of inhibitory interneurons resulting in unrestrained excitatory drive in the neocortex. This alters cortical information processing leading to disease phenotypes. At present, the factors responsible for loss of inhibitory interneurons are unknown, although it has been suggested that selective vulnerability of these populations of cells may be responsible. In cortical dysplasia due to defective migration of specific population of cells, aberrant positioning, rather than loss of inhibitory interneurons, impairs cortical function. Therefore, ameliorating the deficit in migration is an appealing approach to management of CD. We postulate that an optimal level of GABA activity is central to proper migration of GE cells and a reduction, as well as increase, in GABA signaling compromises neuronal migration to contribute to disease phenotype. GABA also plays a multifaceted role during development, including regulation of DNA synthesis and intracellular Ca^{2+} levels; these processes may influence aspects of neuronal migration and their functional integration into circuits. A fascinating, but yet unexplored role of GABA during development, is its involvement in regulating gene transcription to influence proliferation and specification of interneurons. This area of thought deserves greater investigation and exploration. In light of strong evidence linking

alterations of GABA signaling to several neuropsychiatric disorders, an intriguing question/possibility is whether early intervention involving restoring/maintaining an optimum level of GABA activity will reduce the severity and improve treatment outcome.

In the future, it will be interesting to specifically evaluate the migratory properties of the GE cells derived from different regions of the LGE or CGE in our model. In addition, functional analysis of other regions of the neocortex of juvenile ferrets will provide further evidence as to whether similar alterations in IPSCs occur in other regions of the MAM-treated neocortex.

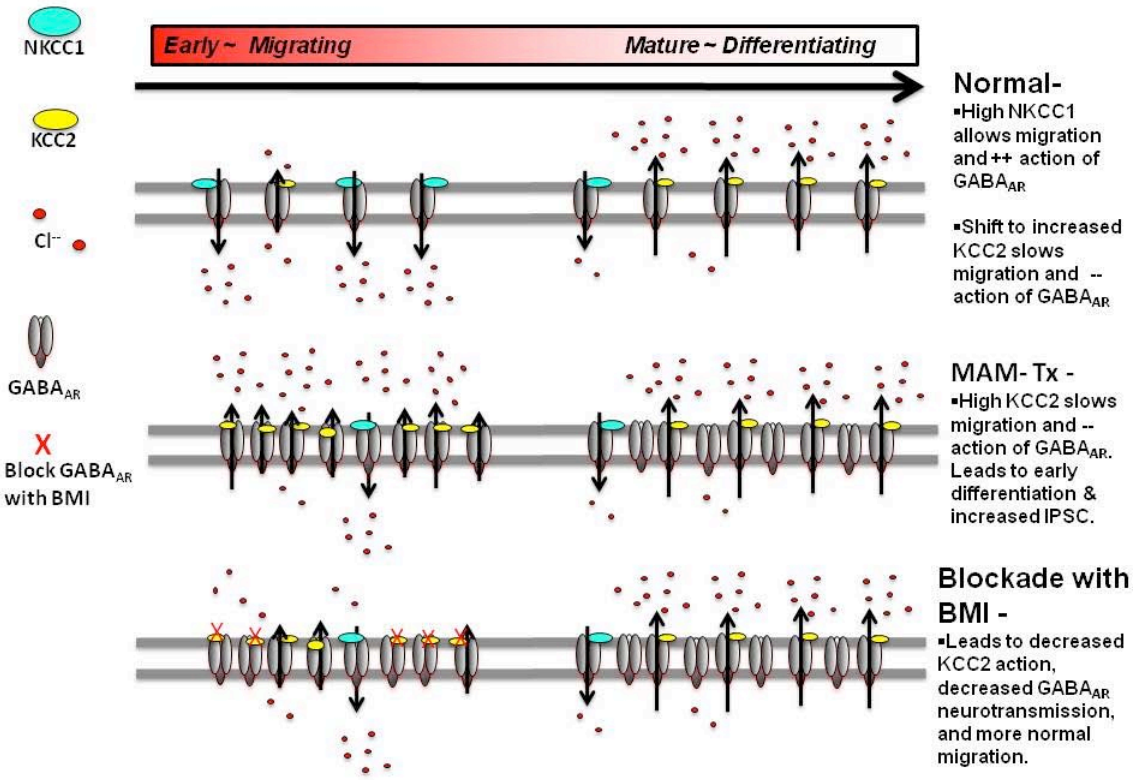

Figure 4: Schematic illustration of the cellular mechanisms contributing to defective migration and increase GABAergic neurotransmission in the MAM model of cortical dysplasia. During development, GABA initially acts as an excitatory neurotransmitter due to outward movement of chloride ion that leads to cell depolarization and cell migration in response to $GABA_{AR}$ activation. As cells matured, increased expression of KCC2 reverses the direction of chloride ion movement to cause depolarization. MAM treatment triggers increased $GABA_{AR}$ activity to promote premature expression of KCC2 to cause deficit in GE cell migration.

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